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The *Saccharomyces cerevisiae rev6-1* Mutation, Which Inhibits Both the Lesion Bypass and the Recombination Mode of DNA Damage Tolerance, Is an Allele of *POL30*, Encoding Proliferating Cell Nuclear Antigen

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ABSTRACT

The *rev6-1* allele was isolated in a screen for mutants deficient for UV-induced reversion of the frameshift mutation *his4-38*. Preliminary testing showed that the *rev6-1* mutant was substantially deficient for UV-induced reversion of *arg4-17* and *ilv1-92* and markedly UV sensitive. Unlike other *REV* genes, which encode DNA polymerases and an associated subunit, *REV6* has been found to be identical to *POL30*, which encodes proliferating cell nuclear antigen (PCNA), the subunit of the homotrimeric sliding clamp, in which the *rev6-1* mutation produces a G178S substitution. This substitution appears to abolish all DNA damage-tolerance activities normally carried out by the *RAD6/RAD18* pathway, including translesion replication by DNA polymerase ζ/Rev1 and DNA polymerase η, and the error-free, recombination-dependent component of this pathway, but has little effect on the growth rate, suggesting that G178S may prevent ubiquitination of lysine 164 in PCNA. We also find that *rev6-1* mutation can be fully complemented by a centromere-containing, low copy-number plasmid carrying *POL30*, despite the presumed occurrence in the mutant of sliding clamp assemblies that contain between one and three G178S PCNA monomers as well as the fully wild-type species.

THE *RAD6/RAD18* DNA damage-tolerance pathway of budding yeast, *Saccharomyces cerevisiae*, promotes the completion of genome replication when nascent strand elongation by the replicase is stalled at sites of template damage. At least two mechanisms may be employed for this purpose: translesion replication by specialized DNA polymerases, a process that is often inaccurate and thus generates mutations, and an accurate process based on recombination between partially replicated sister strands (Zhang and Lawrence 2005). The particular process used is regulated by modification of K164 in PCNA (Hoege *et al.* 2002), the subunit of the homotrimeric sliding clamp that enhances DNA polymerase processivity. K164 is modified by SUMO during normal replication, whereas monoubiquitination of this residue by the Rad6 E2 ubiquitin conjugase present in a Rad6–Rad18 heterodimer promotes translesion replication (Stelter and Ulrich 2003). Polyubiquitination of K164, in which ubiquitin is conjugated to K36 of ubiquitin itself by an Mms2–Ubc13–Rad5 complex, promotes the accurate recombination-dependent process (Ulrich and Jentsch 2000).

Investigation of the translesion replication component of the DNA damage-tolerance pathway has been greatly advanced by the identification of mutants isolated specifically for a reduction in the frequency of induced mutagenesis. These were first isolated in yeast—and in fact in any organism—by Lemontt (1971), producing alleles of genes designated *REV1*, *REV2* (now renamed *RAD5*), and *REV3*. Following this lead, we isolated alleles of *REV4* through *REV7* and *NGM2* (Lawrence *et al.* 1985a,b; Nisson and Lawrence 1986). Of these, *REV3* encodes the catalytic subunit of DNA polymerase ζ (Morrison *et al.* 1989; Nelson *et al.* 1996a), and *REV7* (Torpey *et al.* 1994) encodes an additional subunit of this enzyme. *REV1* encodes a deoxycytidyl transferase activity, but also possesses a more general function in translesion replication (Larimer *et al.* 1989; Nelson *et al.* 1996b, 2000). Although the *rev4* and *rev5* mutations isolated were found on outcrossing to exhibit deficiencies in mutagenesis that were too small for further analysis, *rev5-1* has been found recently (M. Villasmil and P. E. M.Gibbs, unpublished data) to be an allele of *POL32*, which encodes a subunit of DNA polymerase δ, a subunit shown to influence induced mutagenesis (Huang *et al.* 1997, 2000). However, the functions of the two remaining loci, *NGM2* and *REV6*, have not been previously identified.

The *REV6* was a particularly interesting gene for further study because the phenotype of *rev6-1* strains was in several respects similar to that of *rev1*, *rev3*, or *rev7*

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mutants, suggesting that the *REV6* gene product might constitute a hitherto unidentified role in translesion replication. The *rev6-1* allele was isolated in a screen of mutagen-treated cells for strains that exhibited much-reduced frequencies of mutagenesis as measured by the UV-induced reversion of the frameshift allele *his4-38* (Lawrence *et al.* 1985a). Further testing showed that it also reduced the UV-induced reversion frequency of *arg4-17* (ochre) and *ilv1-92* (missense), suggesting that its deficiency for induced mutagenesis, like that of *rev1*, *rev3*, or *rev7* mutants, was likely to be global. Similarly, the *rev6-1* mutant was also found to be sensitive to UV. However, although these phenotypes were similar to the other *rev* mutants, no direct comparison was made. To investigate the function of the *REV6* gene, we cloned and sequenced the *rev6-1* allele and, surprisingly, found that it is an allele of *POL30*, encoding the sliding clamp protein, proliferating cell nuclear antigen (PCNA), in which it produces a G178S substitution. This substitution not only causes a deficiency in translesion replication but also abolishes the error-free, recombination-dependent component of the *RAD6/RAD18* pathway. We suggest that the *rev6-1* allele be renamed *pol30-61*.

MATERIALS AND METHODS

Yeast strains: HSZ1-1C (*MAT*α *rev6-1 arg4-17 his3-*Δ*1 ilv1-92 leu2-3,112 ura3-52*) was used in the screen for a *REV6* clone. Deletions of *REV1*, *REV3*, *RAD30*, and *POL32* were created in CL1265-7C (*MAT*α *arg4-17 his3*Δ-1 *leu2-3,112 trp1 ura3-52*) by the gene-blaster method (Alani *et al.* 1987), and the *rev6-1* allele inserted by gene replacement (Rothstein 1991), to provide strains for assessing the comparative influence of these mutants on UV-induced mutagenesis and survival. The *rev6-1* strain was also used to examine the influence of this mutation on the bypass of specific lesions by translesion replication. HSZY12, a *rad1*Δ derivative of this strain, was used to investigate the effect of the *rev6-1* mutation on lesion bypass employing the error-free recombination process.

Cloning the *REV6* locus and its *rev6-1* allele: A clone carrying the *REV6* locus was isolated by transforming the *rev6-1* strain HSZ1-1C with a yeast genomic library constructed in YCplac 33 (Briggs and Butler 1996), supplied by Scott Butler (University of Rochester School of Medicine and Dentistry), followed by a screen for clones carrying plasmids that complemented the UV sensitivity of the *rev6-1* mutant. To this end, master plates of transformants, together with *REV6* and *rev6-1* controls, on synthetic complete medium lacking uracil were replicated using a rod-type replicator onto a series of plates containing yeast extract peptone dextrose medium that were exposed to 0, 20, 40, 60, 80, and 100 J/m^2 of 254 nm UV and incubated in the dark for 1–3 days. UV-resistant transformants were retested in the same way and levels of *arg4-17* reversion examined by spreading $\sim$4 $\times$ 10^7 cells on each of two plates of synthetic complete medium lacking uracil and arginine, one irradiated with 15 J/m^2 254 nm UV and the other unirradiated, to determine whether they exhibited frequencies of *ARG4* revertants characteristic of *REV6* strains rather than the much lower frequencies found in *rev6-1* mutants. Composition of the media used is as described (Sherman 2002). Sequence analysis of the ends of the genomic insert in a complementing plasmid of this kind indicated that it contained a 12.6-kb segment of chromosome II carrying eight intact open reading frames (ORFs). The subcloning of portions of this 12.6-kb segment identified a 1.83-kb sequence that was fully complementing and that encoded the *POL30* locus on one strand and the YBR089W overlapping ORF on the other strand. All other segments failed to complement. To establish that complementation of the *rev6-1* phenotype depended on *POL30* rather than on YBR089W, the *POL30* cys22 TGT codon was converted to a TGA stop codon, a change that generated a synonymous alteration in the YBR089W reading frame. This substitution was introduced using a Quick-Change (Stratagene, La Jolla, CA) site-directed mutagenesis kit and the following PCR primers: 5′-GGAAATTGACCAA CTGGACTCAATCTTTGAAACCATC-3′ and 5′-GATGGTTTC AAAGATTGAGTCCAGTTGGTCAATTTCC-3′. The identity of the *rev6-1* mutation was determined by recovering the genomic allele using plasmid gap repair (Rothstein 1991) and sequencing the entire gene using Big Dye cycle sequencing and an ABI Genetic Analyzer model 3100. The gapped plasmid was generated by removing the 2.2-kb *Bam*HI/*Hpa*I sequence encompassing the whole *POL30* gene, from a 4-kb *Hin*dIII genomic fragment derived from the original 12.6-kb genomic insert, cloned into YCplac33.

UV mutagenesis, survival, and growth rate: Frequencies of *arg4-17* revertants were estimated by growing each strain at 30° with vigorous shaking for 4 days to late stationary phase in liquid YPD medium and plating $\sim$4 $\times$ 10^7 washed cells on synthetic complete medium without arginine for revertants and suitable dilutions on synthetic complete medium with arginine for estimates of survival. Survival curves were determined by plating washed cells on YPD medium. Plates were irradiated with 2.5, 5.0, 7.5, 10.0, and 12.5 J/m^2 254 nm UV or left unirradiated. All plates were handled under illumination from gold fluorescent lights and incubated at 30° in the dark. Growth rates were examined by inoculating 100 ml of synthetic complete medium, or for transformed strains such medium lacking uracil, in side-arm flasks with 1 $\times$ 10^5 cells of an overnight culture grown in the same medium, followed by incubation at 30° with vigorous shaking. Turbidity of the culture was measured with a Klett-Summerson colorimeter.

Construction of plasmids carrying site specific DNA lesions: Duplex plasmids carrying a T-T *cis–syn* cyclobutane dimer or abasic site uniquely located within a 28-nt single-stranded region, used to estimate frequencies of translesion replication, were constructed as described (Gibbs and Lawrence 1995; Gibbs *et al.* 2005). To produce the dimer-containing species, 36-mers were constructed by ligating an 11-mer carrying this lesion, produced and purified as described (Banerjee *et al.* 1988), to a flanking 12-mer and 13-mer, followed by the annealing of the gel-purified product to an unphosphorylated complementary 28-mer and ligation of this duplex insert into *Eco*RI–*Pst*I-digested pYPOG1, using a two-step process that promoted high ligation efficiency. Following denaturation of the 28-mer, carried out in the presence of a molar excess of its complementary sequence, the gapped circular material was purified by electrophoresis and quantitated by picogreen fluorometry (Molecular Probes, Eugene, OR) using a Turner Quantech fluorometer. Gapped circular constructs containing an abasic site were produced similarly, except that 36-mers carrying a dUMP nucleotide were synthesized and the construct treated after purification with uracil *N*-glycosylase (New England Biolabs, Beverly, MA).

Duplex plasmids carrying specifically located T-T pyrimidinone pyrimidine (6-4) adducts in each strand at staggered positions 28 bp apart, used to investigate the effect of the *rev6-1* mutation on lesion bypass by the error-free recombination process, were constructed as described (Ozgenc *et al.* 2005;

Zhang and Lawrence 2005). To this end, a 72-mer and complementary 80-mer were annealed and ligated into *Eco*RI–*Pst*I-digested pYPOG1 by a method that promoted high yields of the desired product. The complementary 80- and 72-nt strands that form the duplex sequence inserted into either pYPOG1 or pYPOG2 were assembled individually by ligating together oligonucleotides annealed to complementary scaffold oligomers as described (Ozgenc *et al.* 2005), followed by purification of the required single-stranded species by electrophoresis through a 12% denaturing polyacrylamide gel. The 72-mer strand was assembled from the following oligonucleotides in the order given: 5′-TCCACGGTACCTTAG-3′, 5′-GCAAGTTGGAG-3′, 5′-GGTTACCAGTAGCTCGTACCC TC CCCCTTGCGAGTCCAGCAAGACC-3′. For the 80-mer they were 5′-AATTGGTCTTGCGGACTC-3′, 5′-GCAAGTTGGAG-3′, 5′-GGTACGAGCT ACTGGTAACCCTCCCCCTTGCTAAGGT ACCGTGGATGCA-3′. A T-T (6-4) photoadduct was placed at the unique T-T site in the 11-mer 5′-GCAAGTTGGAG-3′ as described (Ozgenc *et al.* 2005) and incorporated into both the 72-mer and the 80-mer. A complete set of strands was also assembled using lesion-free 11-mer to act as controls. Following ligation of the 72-mer/80-mer insert into pYPOG1, covalently closed product was purified by electrophoresis through a 0.7% agarose gel and quantitated with picogreen fluorometry (Molecular Probes) using a Turner Quantech fluorometer.

RESULTS

Isolation of a *REV6* clone: To investigate the molecular role performed by the *REV6* gene, we isolated a clone carrying a wild-type copy of this locus by transforming the *rev6-1* strain HSZ1-1C with a yeast genomic library in the low copy-number plasmid YCplac33 (Briggs and Butler 1996) followed by the screening of transformants for plasmids that complemented the UV sensitivity of the *rev6-1* mutant. One such UV-resistant transformant, found among 4732 screened, contained a yeast genomic sequence from chromosome II spanning nucleotides 414638–427283, which contains eight intact ORFs. Subcloning various parts of this sequence into YCplac33 indicated that a 1.83-kb *Bam*HI/*Bgl*II fragment, but no other segment, fully complemented *rev6-1* UV sensitivity. This region contains the *POL30* gene on one strand and the substantially overlapping YBR089W ORF on the other. Two pieces of evidence show that complementation of the *rev6-1* mutant phenotype depends on a functional *POL30* and is not dependent on YBR089W. First, sequence analysis of the *REV6*/*POL30* allele segregating in the same pedigree as HSZ1-1C revealed that it carried three single-nucleotide polymorphisms compared to the GenBank sequence, two producing synonymous changes in the *POL30* ORF (Phe57, TTC to TTT; Thr89, ACA to ACG) and one outside this ORF. Of these, the Phe57 polymorphism converted the Trp138 TGG of the YBR089W reading frame into a TGA stop codon, which, since the fragment complements, suggests that complementation cannot depend on YBR089W. Second, complementation was abolished when a site-directed change was made in the *POL30* Cys22 codon, converting it from TGT to a TGA stop codon, a change that is synonymous in the YBR089W reading frame. The *rev6-1* mutation therefore encodes a mutant form of PCNA that substantially reduces UV-induced mutagenesis but, since *rev6-1* strains appear to grow at the usual rate, does not seem to materially impair the role of the sliding clamp in normal replication.

To identify sequence alteration present in the *rev6-1* allele, we recovered the *POL30* locus from the *rev6-1* mutant HSZ1-1C by gapped plasmid repair (Rothstein 1991), transforming this strain with pYHS7 from which the *POL30* ORF had been deleted by digestion with *Bam*HI and *Hpa*I. Sequence analysis of the whole of the *POL30* locus in the repaired plasmid showed that it contained a single G:C-to-A:T substitution that produced a glycine178-to-serine substitution. That this single alteration in *POL30* is indeed responsible for the *rev6-1* mutant phenotype was confirmed by the observation that strain HSZY1, created by replacing *POL30* in strain CL1265-7C with the mutant allele (Rothstein 1991), possesses both the UV sensitivity and UV-induced mutation deficiency characteristic of the original *rev6-1* mutant. We conclude that in addition to a K164R substitution, which is known to impair all *RAD6*/*RAD18*-dependent DNA damage-tolerance processes (Hoege *et al.* 2002; Stelter and Ulrich 2003), a single substitution of at least one other residue can impair UV mutagenesis and therefore presumably translesion replication. This finding also raises the questions of whether the *rev6-1* mutation impairs translesion replication by all bypass enzymes in yeast and also whether it decreases the efficiency of the error-free component of the *RAD6*/*RAD18* pathway.

Characterization of the *rev6-1* phenotype: We compared the phenotype of the *rev6-1* mutant to that of isogenic *REV6* strains carrying a *rev3*, *rev1*, *rad30*, or *pol32* deletion by measuring the frequency of *ARG4* revertants induced by irradiation with 254 nm UV and UV sensitivity in each of these strains. Double mutants carrying *rev6-1* and one of these deletions were also examined (Figure 1). Although the frequency of induced *ARG4* revertants was decreased in each of the single mutant strains, and sensitivity to cell killing increased, the extent of these differences varied widely. As shown in Figure 1A, the frequency of *ARG4* revertants in the *rev6-1* strain is much lower than that in the *REV6* strain and is essentially no different from the similarly low frequencies seen in the *rev1*Δ and *rev3*Δ strains. In contrast, the frequency of *ARG4* revertants is decreased to a much smaller extent in the *rad30*Δ and *pol32*Δ strains. The *rev6-1* mutant is also least like the *rad30*Δ and *pol32*Δ strains with respect to survival following exposure to 254 nm UV. As can be seen in Figure 1B, the *rad30*Δ and *pol32*Δ strains, even though slightly more sensitive than the wild-type, are the least sensitive of the mutant strains tested. The *rev6-1* mutant, on the other hand, exhibits much greater sensitivity and is in fact appreciably more sensitive than either of the *rev1*Δ or

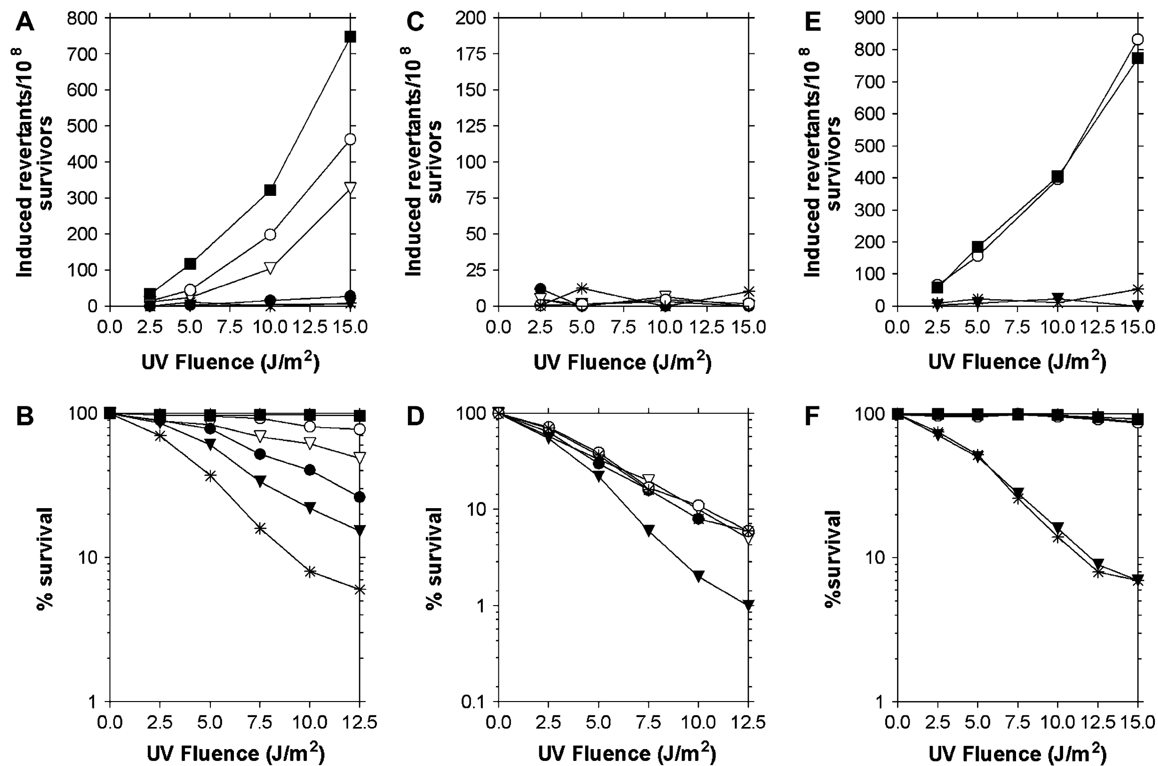

FIGURE 1.—Frequencies of UV-induced reversion of *arg4-17* (A, C, and E) and percentage of survival following UV irradiation (B, D, and F) in *REV6*, *rev6-1*, *rev1*Δ, *rev3*Δ, *rad30*Δ, and *pol32*Δ strains and in *rev6-1 rev1*Δ, *rev6-1 rev3*Δ, *rev6-1 rad30*Δ, and *rev6-1 pol32*Δ double mutants. A–F: ■, *REV6*+; *, *rev6-1*. Additionally, in A and B: ○, *rad30*Δ; ▽, *pol32*Δ; ●, *rev1*Δ; ▼, *rev3*Δ. In C and D: ○, *rev6-1 rev1*Δ; ▽, *rev6-1 pol32*Δ ; ●, *rev6-1 rad30*Δ; ▼, *rev6-1 rev3*Δ. In E and F: ○, *rev6-1* + YCplac33 *POL30*; ▼, *rev6-1* + YCplac33. Data are the average of two or three replicate experiments.

*rev3*Δ strains, perhaps the consequence of impairment of the error-free process. Each of the double mutants exhibits the same very low frequencies of UV-induced *ARG4* revertants as the *rev6-1* single mutant, and the *rev1*Δ, *rad30*Δ, and *pol32*Δ mutants are also each epistatic to the *rev6-1* allele with respect to sensitivity to killing by UV. Interestingly, however, the *rev3*Δ *rev6-1* strain is more UV sensitive than either of the component single mutants, perhaps reflecting the PCNA-independent activities of DNA polymerase ζ.

To investigate the impact of the *rev6-1* mutation on the translesion replication and error-free recombination-dependent components of the *RAD6/RAD18* DNA damage-tolerance pathway more directly, we have transformed a *rev6-1* strain with plasmids carrying specifically located DNA lesions of two types, each designed to examine one of these two processes (GIBBS *et al.* 2005; ZHANG and LAWRENCE 2005). To examine translesion replication, the plasmids carried either an abasic site or T-T *cys–syn* cyclobutane dimer placed centrally within a 28-nt single-stranded region. Plasmid replication, and hence the production of transformants, requires the filling of this gap, which entails translesion replication. The frequency of this event can therefore be estimated by normalizing the number of transformants to the number obtained with an equal amount of lesion-free control plasmid. Because the bypass frequencies of the two lesions are twofold different, we present the data in Table 1 as a percentage of the frequency in the wild type strain, a procedure that makes it easier to compare the contributions of the various gene functions to the bypass of each lesion. As shown in Table 1, the G178S substitution in *rev6-1* essentially abolishes the ability to replicate past both an abasic site and a T-T cyclobutane dimer, indeed to a level lower than that in strains lacking DNA polymerase ζ (*rev3*Δ) or Rev1 (*rev1*Δ) and matched only, at least for the T-T dimer, in strains simultaneously deficient for pol ζ and pol η (*rad30*Δ) or for Rev1 and pol η. Because, as can be seen in Table 1, pol ζ/Rev1 are largely responsible for replication past abasic sites whereas pol η is responsible for replication past the T-T dimer, these results show that the G178S substitution impairs the activity of each of these three enzymes.

In addition to investigating the impact of the *rev6-1* mutation on translesion replication, we also examined its influence on the error-free, recombination-dependent component of the *RAD6/RAD18* pathway. To do this, we transformed an excision-defective (*rad1*Δ) strain and its isogenic *rev6-1* derivative with plasmids that carried a T-T pyrimidinone (6-4) pyrimidine photoadduct [T-T (6-4) photoadduct] in each strand of the duplex, at staggered positions 28 bp apart. C-C mismatches placed

TABLE 1

Bypass frequencies, expressed as percentage of the wild-type frequency, for abasic sites and T-T cyclobutane dimers in *rad30*Δ, *rev3*Δ, *rev1*Δ, *pol32*Δ, and *rev6-1* strains

	Lesion bypass, % of wild-type frequency		
	Abasic site (O)		
Strain	T-O	O-T	T-T *cis–syn* dimer
Wild-type[a]	100.0	100.0	100.0
rev6-1	1.3	0.5	1.3
*rad30*Δ[a]	80.7	87.1	15.3
*rev3*Δ[a]	4.3	5.5	93.6
*rev1*Δ[a]	3.8	2.9	98.5
*rad30*Δ *rev3*Δ[a]	—	—	0.0
*rad30*Δ *rev1*Δ[a]	—	—	1.0
*pol32*Δ[a]	4.7	3.6	112.1

Each lesion is located at the T-T site in the sequence 5′-GCAAGTTGGAG-3′. Actual bypass frequencies and the number of replicate experiments (*N*) for the different lesions in the wild-type strain were: T-O, 32.3% ± 4.2% (*N* = 5); O-T, 24.1% ± 2.8% (*N* = 3); T-T dimer, 60.1% ± 5.8% (*N* = 16).

[a] Data from Gibbs *et al.* (2005).

opposite the T-T (6-4) photoadducts allow the unambiguous identification of the events leading to the completion of replication: recombination between partially replicated sister strands within the interlesion region or translesion replication on one or the other strand. Each event has a unique signature sequence, which in the case of recombination is the presence in replicated plasmids of G:C,G:C and C:G,C:G doublets at the sites previous occupied by the T-T lesions. Although these photoadducts present a severe impediment to translesion replication, an astonishing 54.7% of the plasmids are replicated in the *rad1*Δ strain, with 50.7% of them achieving this by a recombination-dependent process (Zhang and Lawrence 2005) (Table 2). The *rev6-1* mutation reduces this to 19.7% (Table 2), a frequency similar to that found in the strain deleted for *RAD5* (21.9%), a gene whose function is required for the *RAD6*/*RAD18* error-free process. As discussed below and in Zhang and Lawrence (2005), the remainder of recombination occurs by a different process for which the function of Rad52 is required. We conclude that the G178S substitution interferes with two, and perhaps all, of the *RAD6*/*RAD18*-dependent DNA damage-tolerance processes in yeast; it not only abolishes the PCNA-dependent activities of pol ζ, pol η, and Rev1 but also impairs the error-free recombination process.

Although it is clear that processes promoting the replication of lesion-containing DNA templates are defective in *rev6-1* strains, we observed no obvious deficiency in the growth rate of unirradiated cells carrying this mutation, suggesting that G178S PCNA functions reasonably well in the replication of undamaged DNA. To specifically examine this issue, we measured change in absorbance over time in logarithmically growing cultures of isogenic *REV6* and *rev6-1* strains transformed with either YCplac33 *POL30* or YCplac33 (Figure 2). These results show that growth rate in the *rev6-1* mutant is at best only slightly slower than that in the wild-type strain, suggesting that G178S PCNA functions almost normally during replication on undamaged DNA templates.

DISCUSSION

We find that *rev6-1*, a mutation isolated by virtue of its deficiency in UV-induced mutagenesis, is an allele of *POL30* that encodes a G178S substitution in PCNA. This substitution appears to abolish the effective mobilization by the homotrimeric sliding clamp—of which PCNA is the subunit—of pol ζ, pol η, and Rev1 for translesion replication. A comparison of UV-induced reversion frequencies of *arg4-17* and survival in an isogenic series of *rev6-1*, *rev1*Δ, *rev3*Δ, *rad30*Δ, and *pol32*Δ mutant strains (Figure 1) shows that induced reversion is almost completely absent in the *rev6-1* mutant, as it also is in the *rev1*Δ and *rev3*Δ strains, in contrast to the *rad30*Δ and *pol32*Δ strains, which reduce reversion frequencies only modestly. These observations suggest that the G178S substitution in PCNA inhibits translesion replication by pol ζ and Rev1, but do not exclude a similar effect with

TABLE 2

Percentage of total plasmids replicated, percentage replicated by a recombination-dependent process, and percentage replicated using translesion replication in an excision repair-defective (*rad1*Δ) strain and its isogenic *rev6-1*, *rad5*Δ, *rad18*Δ, *rad52*Δ, and *rad18*Δ *rad52*Δ deletion derivatives

Strain	Relevant genotype	% plasmids replicated	% by recombination	% by translesion replication	No. plasmids sequenced	No. replicate experiments
POGY9[a]	*rad1*Δ	54.7 ± 3.2	50.7	4.0	182	5
HSZY12	*rad1*Δ *rev6-1*	22.1 ± 4.6	19.7	2.4	165	5
POGY13[a]	*rad1*Δ *rad5*Δ	23.5 ± 1.9	21.9	1.6	165	5
POGY12[a]	*rad1*Δ *rad18*Δ	15.5 ± 1.6	15.5	0.0	84	6
HSZY9[a]	*rad1*Δ *rad52*Δ	28.6 ± 3.6	24.2	4.4	169	5
HSZY7[a]	*rad1*Δ *rad18*Δ *rad52*Δ	2.3 ± 0.8	2.3	0.0	22	11

[a] Data from Zhang and Lawrence (2005).

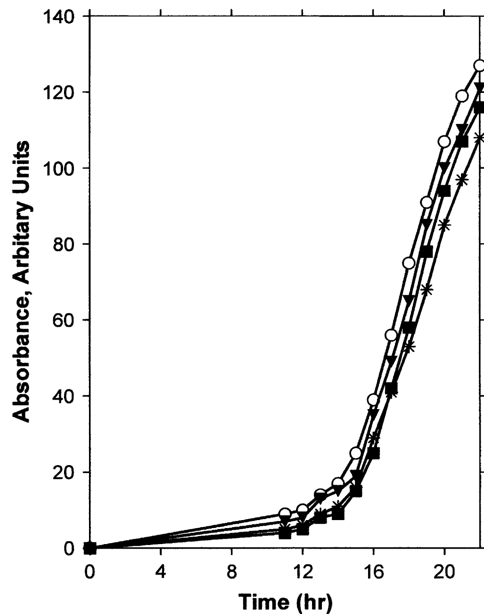

FIGURE 2.—Growth rate monitored by turbidity (arbitrary absorbance units) and viable cell titer in *REV6*+ and *rev6-1* strains transformed with either YCplac33 *POL30* or YCplac33. ■, *REV6*; *, *rev6-1*; ○, *rev6-1* with YCplac33 *POL30*; ▼, *rev6-1* with YCplac33. Data are the average of three experiments.

pol η. In keeping with this conclusion, UV-induced reversion of *arg4-17* is essentially absent in double mutants carrying *rev6-1* and one of the deleted genes. The *rev6-1* mutant is also more sensitive to killing by UV than the other strains and in fact is more sensitive than the *rev3*Δ strain, consistent with the G178S alteration impairing a DNA damage-tolerance process other than translesion replication, as discussed below. All but one of the double mutants is epistatic to *rev6-1* with regard to UV sensitivity. The *rev6-1 rev3*Δ strain, however, is more sensitive than either single mutant, perhaps because pol ζ promotes survival in processes other than those concerned with DNA damage tolerance or those dependent on PCNA.

The conclusion that the G178S abolishes virtually all capacity for translesion replication was also supported by transforming a *rev6-1* strain with plasmids that carried either an abasic site or T-T dimer within a 28-nt single-stranded region of an otherwise duplex vector, which provides a direct estimate of lesion bypass frequencies. We chose a T-T dimer because it is bypassed *in vivo* almost exclusively by pol η; although, as shown in Table 1, dimer bypass can be performed by pol ζ/Rev1 when pol η is absent, in its presence they do so only in ≤0.6% of bypass events (GIBBS *et al.* 2005). An abasic site, conversely, is bypassed predominantly by pol ζ/Rev1. Bypass frequencies for both of these lesions were no more than ~1% of the wild type frequency in the *rev6-1* mutant, suggesting that the G178S substitution abolishes the activity of both polymerases in translesion replication. Regarding the dimer, such a result is similar to what is seen in the *rad30*Δ *rev3*Δ and *rad30*Δ *rev1*Δ strains but is unlike the bypass frequency in the *pol32*Δ mutant, which is at wild-type level. This difference between *rev6-1* and *pol32*Δ strains suggests that unlike Pol32, which appears to influence mutagenesis indirectly via interactions with Srs2 or checkpoint proteins (GIBBS *et al.* 2005), the G178S substitution influences lesion bypass DNA polymerases more directly.

In addition to abolishing translesion replication, the PCNA G178S substitution in the *rev6-1* strain also inhibits the error-free, recombination-dependent component of the *RAD6/RAD18* pathway, presumably the reason for the greater sensitivity to UV of the *rev6-1* strain compared to the *rev3*Δ mutant. Although 50.7% of the plasmids carrying T-T (6-4) photoadducts completed replication by a recombination-dependent mechanism in the *rad1*Δ control strain (Table 2), only 19.7% did so in the *rad1*Δ *rev6-1* strain. The latter value is similar to the 21.9% observed in the *rad1*Δ *rad5*Δ strain, suggesting that the G178S substitution essentially abolishes the recombination-dependent component of the *RAD6/RAD18* pathway for which *RAD5* function is essential. The remaining fraction of plasmids using a recombination mechanism to complete replication in the *rad1*Δ *rev6-1* and *rad1*Δ*rad5*Δ strains was generated by a different process, which is not part of the *RAD6/RAD18* pathway, and instead uses *RAD52*. As the very small fraction (2.3%) of plasmids replicated in the *rad1*Δ *rad18*Δ *rad52*Δ strain shows (Table 2), the *RAD18*- and *RAD52*-dependent processes between them account for nearly all of the recombination observed in the *rad1*Δ control strain. Approximately 60–70% can be ascribed to the *RAD6/RAD18*-dependent process, with the remaining 30–40% resulting from the *RAD52*-dependent mechanism (ZHANG and LAWRENCE 2005). Although all of the strains used in these experiments carry a *RAD1* deletion, and this gene is known to be involved in certain types of recombination (RATTRAY and SYMINGTON 1995), there is no obvious reason why its absence should influence template strand switching, although it may well influence the *RAD52*-dependent process.

The *rev6-1* mutant therefore appears to be deficient in both the translesion replication and the error-free, recombination-dependent components of the *RAD6/RAD18* DNA damage-tolerance pathway. At the same time, unirradiated cells of this mutant grow at a rate that is little different from that of the wild type (Figure 2), resulting in a phenotype that is remarkably similar to that of the PCNA K164R mutation. The G178 residue is located in a β-sheet at the junction between the monomers composing the sliding clamp, and the side chain of a substituted serine at this site appears to show an unfavorable steric interaction with Y114 located in the adjacent monomer. The PCNA G178S substitution may therefore inhibit modification of K164, perhaps by changing the structure of the sliding clamp sufficiently

to prevent binding of the Rad6/Rad18 heterodimer that monoubiquitinates this residue. Alternatively, the altered structure may prevent the association of the sliding clamp with other proteins required for the activities carried out by the *RAD6/RAD18* pathway.

Surprisingly, the *rev6-1* mutation is fully recessive to the *POL30* wild-type allele, with respect to both survival and UV-induced reversion of *arg4-17* (Figure1, E and F), and can be fully complemented by a centromere-containing, low copy-number plasmid carrying the *POL30* gene, although not by the vector alone, despite the presumed occurrence in the mutant of sliding clamp assemblies that contain anywhere between one and three G178S PCNA monomers as well as the fully wild-type species. Such complementation is in marked contrast to the dominant negative phenotype observed in heterozygous diploids carrying alleles encoding either the ED104,105AA or the DE256,257AA alterations together with the wild type (Torres-Ramos *et al.* 1996). The observation that the growth rate of the *rev6-1* mutant is not appreciably different from that of the wild type (Figure 2) suggests that its complementation by the *POL30* gene is not the consequence of the instability—and thus a higher turnover rate—of clamp assemblies that contain G178S PCNA, resulting in a largely wild-type clamp being used for almost all replication. Perhaps the presence of even a single wild-type PCNA monomer may be sufficient to allow normal function of the *RAD6/RAD18* pathway, either because clamps containing mutant and wild type PCNA assume a fully normal structure or because association is intrinsically confined to a single monomer.

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