# C-Terminal Flap Endonuclease (*rad27*) Mutations: Lethal Interactions With a DNA Ligase I Mutation (*cdc9-p*) and Suppression by Proliferating Cell Nuclear Antigen (*POL30*) in *Saccharomyces cerevisiae*

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## ABSTRACT

During lagging-strand DNA replication in eukaryotic cells primers are removed from Okazaki fragments by the flap endonuclease and DNA ligase I joins nascent fragments. Both enzymes are brought to the replication fork by the sliding clamp proliferating cell nuclear antigen (PCNA). To understand the relationship among these three components, we have carried out a synthetic lethal screen with *cdc9-p*, a DNA ligase mutation with two substitutions (F43A/F44A) in its PCNA interaction domain. We recovered the flap endonuclease mutation *rad27-K325** with a stop codon at residue 325. We created two additional *rad27* alleles, *rad27-A358** with a stop codon at residue 358 and *rad27-pX8* with substitutions of all eight residues of the PCNA interaction domain. *rad27-pX8* is temperature lethal and *rad27-A358** grows slowly in combination with *cdc9-p*. Tests of mutation avoidance, DNA repair, and compatibility with DNA repair mutations showed that *rad27-K325** confers severe phenotypes similar to *rad27Δ*, *rad27-A358** confers mild phenotypes, and *rad27-pX8* confers phenotypes intermediate between the other two alleles. High-copy expression of *POL30* (PCNA) suppresses the canavanine mutation rate of all the *rad27* alleles, including *rad27Δ*. These studies show the importance of the C terminus of the flap endonuclease in DNA replication and repair and, by virtue of the initial screen, show that this portion of the enzyme helps coordinate the entry of DNA ligase during Okazaki fragment maturation.

CELLULAR maintenance of genomic integrity is essential for the continued viability of all organisms. The fidelity of DNA replication has to be maintained and DNA insults have to be repaired to ensure that deleterious mutations are not passed on to progeny or cause cancerous growth. A number of cellular proteins have multiple roles in DNA replication, mutation avoidance, and repair. In *Saccharomyces cerevisiae*, the flap endonuclease, proliferating cell nuclear antigen (PCNA), and DNA ligase I encoded by *RAD27*, *POL30*, and *CDC9*, respectively, are all required for proper replication and also function to avoid mutation and to facilitate repair.

The flap endonuclease, FEN-1 in humans, is a highly conserved structure-specific nuclease that has both endonuclease and 5′–3′ exonuclease activity. During lagging-strand replication these activities function to remove primers from Okazaki fragments, either by endonucleolytic cleavage of a flap made by strand displacement (LIU *et al.* 2004) or by sequential exonucleolytic removal of single nucleotides at the 5′ end of the primer (MURANTE *et al.* 1994).

While deletion of *RAD27* is not lethal to yeast cells, the *rad27Δ* mutant exhibits temperature-sensitive growth, is a mutator, and undergoes genomic instability (JOHNSON *et al.* 1995; REAGAN *et al.* 1995; TISHKOFF *et al.* 1997b; CHEN and KOLODNER 1999). In addition, its sensitivity to low doses of the methylating agent methylmethane sulfonate (MMS) implicates the participation of the enzyme in base excision repair (BER) (REAGAN *et al.* 1995; WU and WANG 1999). *rad27Δ* mutants have been reported to be either mildly sensitive to UV light or not sensitive to UV light (REAGAN *et al.* 1995; SOMMERS *et al.* 1995). In the strain background that the mutant is mildly sensitive, its combination with *rad2Δ* yields a double mutant more sensitive than each single mutant, implying that the enzyme does not participate in *RAD2*-mediated nucleotide excision repair (NER) (REAGAN *et al.* 1995). The flap endonuclease has also been implicated in double-strand break (DSB) repair by virtue of the incompatibility of *rad27Δ* with mutations of the DSB repair pathways (TISHKOFF *et al.* 1997b; SYMINGTON 1998). In addition, either the yeast enzyme or its human ortholog has been shown to participate in reactions of homologous recombination, nonhomologous end joining, and telomere maintenance (PARENTEAU and WELLINGER 1999, 2002; WU *et al.* 1999; WANG *et al.* 2004; KIKUCHI *et al.* 2005). Curiously, the *rad27Δ* mutant is not sensitive to gamma radiation but is sensitive to

high doses of MMS that are thought to act as a radiomimetic agent (Reagan *et al.* 1995; Sommers *et al.* 1995).

PCNA is the replicative clamp that acts as a scaffold to facilitate the loading of DNA replication and repair proteins, including DNA ligase I and the flap endonuclease to DNA (Warbrick 2000, 2006; Maga and Hubscher 2003). PCNA (*POL30*) is essential for cell viability, which is indicative of its central role in DNA metabolism. Biochemical characterization of its effect on the flap endonuclease shows that it stimulates its activity ~50-fold, evidencing the productive nature of the interaction (Gomes and Burgers 2000; Tom *et al.* 2000; Frank *et al.* 2001; Stucki *et al.* 2001). The ability of DNA ligase to efficiently catalyze the formation of phosphodiester bonds in the DNA backbone may also be facilitated by its binding to PCNA. Tom *et al.* (2001) showed that, *in vitro*, PCNA enhances the ligation reaction 5-fold and that the stable association of DNA ligase with nicked duplex DNA requires PCNA.

Both DNA ligase and the flap endonuclease bind to PCNA via their respective PCNA interactive peptide domains (PIP box). The PIP box is a conserved sequence motif of the amino acids QXXLXXFF. The PIP box fits into the interdomain connector loop (IDCL) of PCNA to provide a protein–protein interaction surface (Gomes and Burgers 2000; Chapados *et al.* 2004; Sakurai *et al.* 2005; Pascal *et al.* 2006). Mutations in the PIP box or the IDCL that impair the interaction of DNA ligase and the flap endonuclease to PCNA lead to genomic instability (Amin and Holm 1996; Eissenberg *et al.* 1997; Gary *et al.* 1999; Refsland and Livingston 2005; Subramanian *et al.* 2005). We have reported that the double mutants made by combinations of *cdc9-p*, *rad27-p*, and *pol30-90*—mutations with alterations of the PIP box or the IDCL in the respective proteins—have synergistic phenotypes with respect to MMS sensitivity and to trinucleotide repeat instability (Refsland and Livingston 2005). These results suggest that the two enzymes function in a concerted manner that is facilitated by PCNA.

The precise nature of how PCNA coordinates the entry of the flap endonuclease and DNA ligase into the replication fork is not well understood. Biochemical and structural studies have begun to elucidate a possible ordering of these PCNA-mediated interactions. The possibility of such an ordering is underscored by the observation that DNA ligase adopts a toroidal conformation by completely encircling duplex DNA while interacting with PCNA (Pascal *et al.* 2004). Moreover, both PCNA and DNA ligase may be loaded onto the DNA in a mechanism utilizing the replication clamp loader replication factor C (RFC) (Levin *et al.* 2004; Vijayakumar *et al.* 2009), again suggesting a complete encirclement of the DNA by DNA ligase as well as by PCNA. PCNA and DNA ligase are similar in size and their interaction is likely to extend along the face of PCNA in a manner that would prevent other proteins such as the flap endonuclease from binding to the IDCL (Pascal *et al.* 2004, 2006). A biochemical study with purified yeast proteins showed that the two enzymes cannot bind simultaneously to PCNA (Subramanian *et al.* 2005). These studies suggest that a coordinated sequential interaction among PCNA, DNA ligase, and the flap endonuclease is important for replication and repair.

Alternatively, both the flap endonuclease and DNA ligase may bind to the same molecule of PCNA. Since PCNA is a homotrimer, DNA ligase can potentially bind to one monomer while the flap endonuclease binds to another, using its extended C-terminal tail in a conformation allowing it to be tethered to PCNA concurrently with DNA ligase (Gomes and Burgers 2000; Sakurai *et al.* 2005). DNA ligase could also bind to PCNA in an extended conformation while the flap endonuclease cleaves the DNA. *Sulfolobus solfataricus* DNA ligase has been shown to have an open, extended conformation while binding to PCNA (Pascal *et al.* 2006). Presumably, once the flap endonuclease has removed the 5′ flap, DNA ligase acquires a closed, ring-shaped conformation to catalyze the joining of Okazaki fragments (Pascal *et al.* 2006).

Exactly how the interaction of these enzymes with PCNA is coordinated *in vivo*, whether singly or concurrently, is not well understood. To further elucidate how the interaction of DNA ligase with PCNA is ordered, we performed a genetic screen to identify mutations that are synthetically lethal with *cdc9-p* (F44A/F35A), an allele of DNA ligase that has impaired binding to PCNA (Refsland and Livingston 2005; Subramanian *et al.* 2005). We postulated that genes recovered from this screen would function in DNA repair, replication, and recombination or would be involved in ordering the DNA ligase–PCNA interaction. From the screen we recovered a truncated allele of *RAD27*, *rad27-K325**. This allele encodes a protein that lacks the PIP box and the entire C-terminal domain of the enzyme but retains the N terminus containing the nuclease activities. We have characterized this allele and compared it to two other *rad27* alleles in which we have created different alterations of the C-terminal end of the flap endonuclease.

## MATERIALS AND METHODS

**Yeast strains:** All *S. cerevisiae* strains used in this study, with the exception of SSL938, are isogenic to SSL204 (*ura3 ade2 his3 trp1 leu2*) and are listed in Table 1 (Dornfeld and Livingston 1991). Only differences between this strain and the derived strains are noted. Deletion strains were created in this background by single-step disruption (Rothstein 1983) and allelic substitutions were made using two-step transplacement (Scherer and Davis 1979). In some cases mutations vital to the synthetic-lethal screen, *e.g.*, *ura3* and *ade2*, are shown for clarity. Strain SSL938 was derived from *ura3*, *his3*, *trp1*, and *CAN1* strains in our collection that did not contribute to the SSL204 lineage and was made *rad27Δ* by single-step disruption.

**TABLE 1**

**Yeast strains**

| Strain | Genotype | Source |
|---|---|---|
| SSL204 | his3Δ200 trp1 leu2 ade2-101 ura3-52 | DORNFELD and LIVINGSTON (1991) |
| SSL254 | MATa lys5Δ::HIS3 | This study |
| SSL533 | MATa rad27Δ::HIS3 | SCHWEITZER and LIVINGSTON (1998) |
| SSL534 | MATα rad27Δ::HIS3 | SCHWEITZER and LIVINGSTON (1998) |
| SSL535 | MATa rad2Δ::URA3 | This study |
| SSL539 | MATa exo1Δ::URA3 | This study |
| SSL543 | MATa pol30-79 | SCHWEITZER and LIVINGSTON (1999) |
| SSL544 | MATa pol30-90 | SCHWEITZER and LIVINGSTON (1999) |
| SSL545 | MATα pol30-90 | SCHWEITZER and LIVINGSTON (1999) |
| SSL612 | MATa cdc9-1 | IRELAND et al. (2000) |
| SSL615 | MATa dna2-1 | IRELAND et al. (2000) |
| SSL630 | MATa cdc9Δ::KanMX {pRS316.CDC9} | This study |
| SSL631 | MATα cdc9Δ::KanMX {pRS316.CDC9} | This study |
| SSL632 | MATa cdc9-p | REFSLAND and LIVINGSTON (2005) |
| SSL633 | MATα ade3Δ::hisG | This study |
| SSL634 | MATa ade3Δ::hisG cdc9-p | This study |
| SSL635 | MATα ade3Δ::hisG cdc9-p | This study |
| SSL636 | MATa ade3Δ::hisG cdc9-p lys5Δ::HIS3 | This study |
| SSL637 | MATα ade3Δ::hisG cdc9-p lys5Δ::HIS3 | This study |
| SSL638 | MATa ade3Δ::hisG cdc9Δ::KanMX {pRS316.CDC9} lys5Δ::HIS3 | This study |
| SSL639 | MATα ade3Δ::hisG cdc9Δ::KanMX {pRS316.CDC9} lys5Δ::HIS3 | This study |
| SSL642 | MATa FUR1::HIS3::FUR1 | This study |
| SSL643 | MATα FUR1::HIS3::FUR1 | This study |
| SSL644 | MATa ade3Δ::hisG cdc9-p FUR1::HIS3::FUR1 | This study |
| SSL651 | MATα ade3Δ::hisG cdc9-p FUR1::HIS3::FUR1 | This study |
| SSL649 | MATa rad27-p | REFSLAND and LIVINGSTON (2005) |
| SSL650 | MATα rad27-p | REFSLAND and LIVINGSTON (2005) |
| SSL667 | MATα cdc9-p rad27-p | REFSLAND and LIVINGSTON (2005) |
| SSL826 | MATa rad51Δ::ADE2 | ASLESON and LIVINGSTON (2003) |
| SSL927 | MATa rad27-A358*-isolate1 | This study |
| SSL928 | MATa rad27-A358*-isolate2 | This study |
| SSL929 | MATa rad27-pX8-isolate1 | This study |
| SSL930 | MATa rad27-pX8-isolate2 | This study |
| SSL931 | MATa cdc9-p rad27-pX8 | This study |
| SSL933 | MATa cdc9-p rad27-A358* | This study |
| SSL935 | MATa rad27-K325*-isolate1 | This study |
| SSL936 | MATα rad27-K325*-isolate2 | This study |
| SSL938 | MATa rad27Δ::HIS3 | This study |
| SSL944 | MATa exo1Δ::URA3 rad27-pX8 | This study |
| SSL955 | MATa exo1Δ::URA3 rad27p | This study |
| SSL957 | MATa exo1Δ::URA3 rad27-358* | This study |

**Media:** Media were prepared according to standard recipes (BURKE et al. 2000) except that synthetic media were buffered with succinic acid–NaOH as previously described (HARTWELL 1970). In particular, the omission media in these studies were made from a single batch of nutrients that were mixed from solid ingredients in the proportion previously described (BURKE et al. 2000) but lacked uracil, leucine, and tryptophan. When necessary, these were added in the amount previously specified (BURKE et al. 2000).

**Synthetic lethal screen:** To screen for mutations that are synthetically lethal in combination with cdc9-p, a cdc9-p strain was modified to make it both ade3 and FUR1::HIS3::FUR1. Tester strains were also constructed to test for dominance (cdc9-p ade3Δ::hisG lys5::HIS3) and to test for inactivating mutations in CDC9 (cdc9Δ::KanMX {pRS316.CDC9.ADE3} ade3Δ::hisG lys5::HIS3).

The synthetic lethal screen was carried out using a red/white colony-sectoring assay in an ade2 ade3 background (KRANZ and HOLM 1990). The strain was modified by duplicating FUR1 to eliminate false positive mutations (fur1) in uracil utilization (KOREN et al. 2003). The modified cdc9-p strain was transformed with the plasmid pRS316.ADE3.CDC9. Approximately 300 transformed cells were spread on YPD media and mutagenized by exposure to 3.4 mW/cm$^2$ of UV light (253.7 nm UV-C) to 60% survival. Cells that grew at 30° and did not appear to contain white sectors were streaked onto agar containing 5-FOA. Those that proved to require pRS316.ADE3.CDC9 for survival were tested for dominance and for the absence of inactivating mutations of CDC9. The tester strain for dominance could also be used to test the segregation pattern of the synthetic lethal mutation. At this point candidates that were recessive and segregated as a single

mutation were outcrossed to the wild-type parent to recover the synthetic lethal mutation in a *CDC9* background. Of the candidates that were recessive and segregated as a single mutation, we found two that also conferred temperature-sensitive growth at 35°. Because mutations in *POL30* (PCNA) might have caused a synthetic lethal phenotype and temperature-sensitive growth, we transformed the two candidates with pRS316.*POL30*. One candidate grew better when transformed, and we chose this candidate for further study.

**Identification of the synthetic lethal mutation:** To identify the mutation causing synthetic lethality with *cdc9-p*, we transformed the outcrossed mutant with a yeast genomic library (Jauert *et al.* 2005). We recovered plasmids with the genomic fragment containing *APN1*, *RAD27*, and *ABF1*. We took a candidate gene approach and transformed the isolate with pRS316.*RAD27*, which rescued the temperature lethality. The *rad27* allele from the candidate was recovered by PCR and subjected to DNA sequencing. The copy had a stop codon at amino acid residue 325 in *RAD27*.

**rad27 alleles:** We recreated the *rad27* mutation, *rad27-K325*\*, that we had recovered, in our wild-type strain along with a flanking restriction enzyme recognition sequence that did not further alter the gene. We also made two additional alleles, *rad27-A358*\* and *rad27-pX8* (339QGRLDGFF346–339AAAAKAAA346) by the same methods. Because of the propensity of *rad27* mutations to undergo genetic changes, each of our mutations as well as *rad27Δ* was backcrossed to our wild-type parental strain. The resulting diploids were sporulated, and only strains that yielded a majority of complete tetrads were used. Mutant isolates were confirmed by PCR and restriction digestion. Two isolates (spore colonies) of each mutation were used in our studies, although only one is shown.

**Mutation avoidance assays:** The rate of spontaneous forward mutation to canavanine resistance was measured in the *rad27* mutants with or without the PCNA overexpression plasmids, pRS424.*POL30* or pRS425.*POL30*. The canavanine mutagenesis assay was modified from methods described previously (Marsischky *et al.* 1996; Sia *et al.* 1997). At least three independent experiments were performed for each strain. Mutation rates were estimated using the method of the median (Lea and Coulson 1949). Statistical differences were determined by averaging three or more rate estimations and comparing values using Student's *t*-test.

To measure the rate of dinucleotide repeat instability, plasmid pSH44 was introduced into our strains, grown in media selective for tryptophan, and plated to medium containing 5-FOA as previously described (Henderson and Petes 1992; Kokoska *et al.* 1998). Mutation rates were estimated using the method of the median (Lea and Coulson 1949). Statistical differences were determined by averaging three or more rate estimations and comparing values using Student's *t*-test.

**MMS sensitivity:** All *rad27* strains with or without pRS424.*POL30* or pRS426.*POL30*, and *cdc9-p rad27-p* with or without pRS426.*rad27-K325*\*, were tested for MMS resistance at 25° on the basis of the method previously described (Boundy-Mills and Livingston 1993). The means of at least three independent experiments were used to generate survival curves.

**Growth capability:** The temperature sensitivity of the *rad27*, *cdc9-1*, and *dna2-1* strains with or without pRS424.*POL30* or pRS426.*rad27-K325*\* was examined by performing spotting assays. Log phase cultures (OD$_{600}$ ∼ 0.6–1) were grown in appropriate media and serially diluted in a 96-well microtiter dish. Spots were made on media such that the highest-density spot contained 15,000 cells with 10-fold decreases in the following three consecutive spots. The plates were incubated at 25° and 35° for 4 days unless otherwise specified.
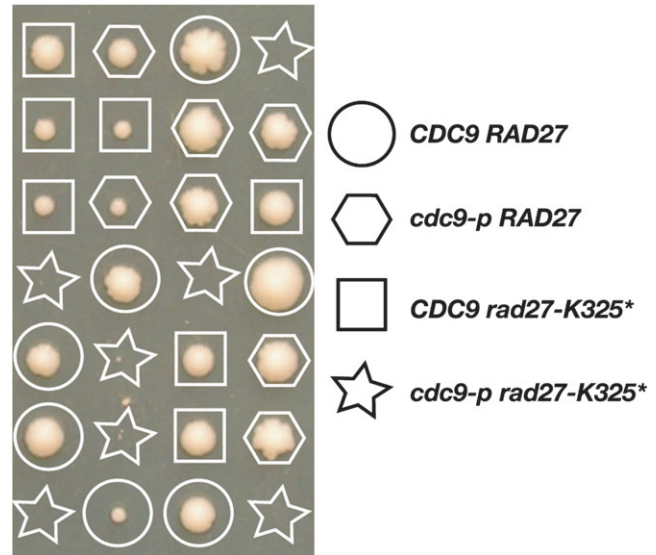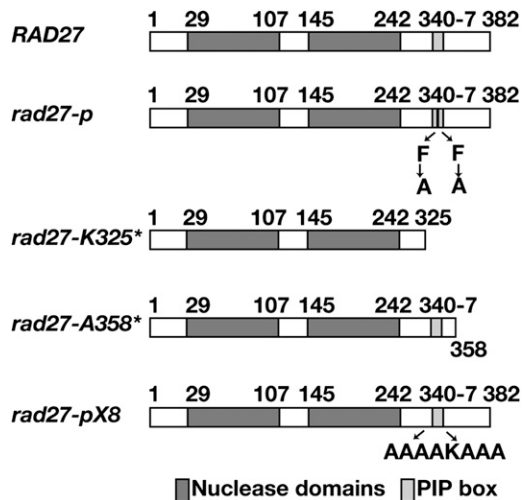


Figure 1.—Synthetic lethality of *rad27-K325*\* combined with *cdc9-p* (F43A/F44A). A diploid heterozygous for both mutations was sporulated, and the spore colonies were germinated on YPD agar at 25°. The genotypes of viable spore colonies were determined by restriction enzyme digestion of PCR products of the *RAD27* and *CDC9* alleles.

## RESULTS

**Identification of *rad27-K325*\* in a synthetic lethal screen with *cdc9-p*:** We screened for mutations that confer synthetic lethality with the DNA ligase I mutation *cdc9-p* that has substitutions of the two aromatic residues of the enzyme's PIP box (F43A/F44A). The *cdc9-p* mutant does not exhibit growth defects, but the mutated copy of DNA ligase I binds weakly to PCNA and causes instability of CAG repeat tracts (Refsland and Livingston 2005; Subramanian *et al.* 2005). To recover mutations that are synthetically lethal with *cdc9-p*, we introduced a wild-type copy of *CDC9* on a *URA3 ADE3*-containing plasmid into the *cdc9-p* strain, mutagenized it, and screened for colonies that could not sector or grow on agar containing 5-FOA. We next screened the isolates for additional phenotypes that might suggest an involvement in DNA replication or repair. One isolate was temperature sensitive for growth and partially suppressible by high-level expression of PCNA (*POL30*) on YPD. Rescue of the temperature sensitivity with a yeast genomic library identified clones containing *RAD27* encoding the flap endonuclease. DNA sequencing of the *RAD27* and *POL30* alleles in the isolate revealed a stop codon at position 325 in *RAD27* and no mutations in *POL30*. The *rad27* mutation *rad27-K325*\* was recreated and used to replace the wild-type copy in our parental wild-type strain. To authenticate that *rad27-K325*\* is sufficient to yield the synthetic lethal phenotype with *cdc9-p*, the *rad27-K325*\* allele was mated to the parental *cdc9-p* strain and the diploid was sporulated. The spore colonies (Figure 1) show that each single mutant is able
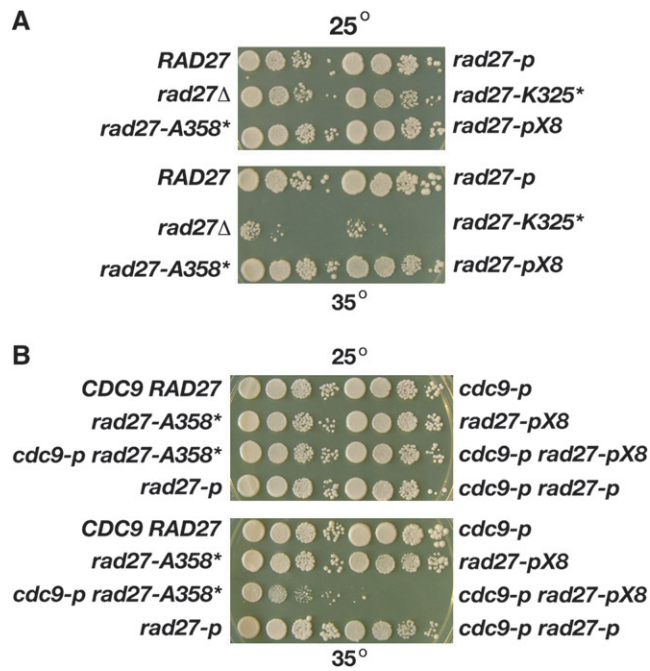
FIGURE 2.—*rad27* alleles.



FIGURE 3.—Temperature-sensitive growth of *rad27* mutants. (A) Growth of mutants at 25° and 35° on YPD agar. (B) Growth of *cdc9-p rad27* double mutants at 25° and 35° on YPD agar.

to germinate and to form colonies, but the *rad27-K325* *cdc9-p* double mutant cannot.

Because we already found a genetic interaction between *cdc9-p* and *rad27-p* (REFSLAND and LIVINGSTON 2005), the corresponding PIP box mutations of the aromatic residues of the flap endonuclease (F346A/F347A) (GARY *et al.* 1999) and the identification of an additional *rad27* allele that disturbs the axis among the flap endonuclease, DNA ligase, and PCNA warranted further examination. The position of the stop codon in *rad27-K325* leaves the nuclease domains of the flap endonuclease intact but removes other domains. The deleted domains include (1) a hinge region that permits rotation of PCNA-bound flap endonuclease to reach the DNA, (2) the PIP box, (3) residues C-terminal to the PIP box that bind to PCNA, and (4) additional C-terminal domains that may be necessary for nuclear import, protein–protein interactions, and post-translational modifications (LIU *et al.* 2004) (Figure 2). Co-crystallization of the human flap endonuclease with PCNA showed that the enzyme makes contacts with PCNA through a number of its domains. The flap endonuclease makes contacts with PCNA through its core domain, through residues N-terminal to the PIP box, through the PIP box, and through residues C-terminal of the PIP box (SAKURAI *et al.* 2005). The most extensive interactions are between the PIP box of the flap endonuclease and the hydrophobic pocket of the IDCL of PCNA and between C-terminal residues of the flap endonuclease and additional residues of the IDCL. To better understand how these regions of the flap endonuclease contribute to its interaction with PCNA, we made two additional *rad27* alleles. One allele substitutes α-helical forming residues for the eight amino acids of the PIP box (*rad27-339AAAAKAAA346*, named *rad27-pX8*) and the other truncates the enzyme beyond the C-terminal residues that interact with the IDCL of PCNA (*rad27-A358*) (Figure 2). These alleles, as well as the complete deletion allele *rad27Δ* and the original flap

endonuclease PIP box allele *rad27-p*, were used for subsequent characterizations and comparisons.

**Phenotypic characterizations of *rad27* alleles:** To learn whether these *rad27* mutations possess unique phenotypes and to distinguish one from another and from the null mutation, we carried out assays for growth, mutagenesis, and DNA repair. Because *rad27* mutants are genetically unstable, our characterizations were carried out with two isolates of each mutant, although only one is shown. In all tests the two isolates had the same phenotype, including statistical differences when calculated.

To begin, we determined whether the strains are temperature sensitive for growth at 35°. Both *rad27Δ* and *rad27-K325* exhibit temperature-sensitive growth whereas *rad27-A358* and *rad27-pX8* grow robustly at 35° on YPD (Figure 3A). Thus, the loss of the C terminus that eliminates all PCNA interacting residues (save for the few interactions between the core of the enzyme and PCNA) is more deleterious than the other mutations that retain either the PIP box (*rad27-A358*) or the residues that interact with the hydrophobic pocket of the IDCL (*rad27-pX8*).

While neither *rad27-pX8* nor *rad27-A358* confer temperature-sensitive growth as single mutations, *rad27-pX8* exhibits temperature-sensitive growth as a double mutant with *cdc9-p*, and *rad27-A358* confers slow growth at the restrictive temperature when combined with *cdc9-p* (Figure 3B). This also contrasts them with the *rad27Δ* and *rad27-K325* mutants that have an absolute lethality with

TABLE 2

**Mutation rates in *rad27* mutants**

| Strain | Mutation rate ($\times 10^{-7}$ events/cell/generation) $\pm$ SD[a,b] |
|---|---|
| *RAD27* | 1.1 ± 0.7 (1) |
| *rad27-p* | 1.2 ± 0.6 (1) |
| *rad27Δ* | 129 ± 77 (117) |
| *rad27-K325** | 165 ± 50 (150) |
| *rad27-A358** | 3.1 ± 1 (3) |
| *rad27-pX8* | 18 ± 7 (17) |

Mutation to canavanine resistance for cells grown in YPD media is shown.

[a] SD, standard deviation.

[b] Values normalized to the *RAD27* rate are shown in parentheses. All the *rad27* mutants, apart from *rad27-p*, have mutation rates significantly higher ($P < 0.05$) than the wild-type rate.

TABLE 3

**Dinucleotide repeat instability in *rad27* alleles**

| Strain | Mutation rate ($\times 10^{-6}$ events/cell/generation) $\pm$ SD[a,b] |
|---|---|
| *RAD27* | 1 ± 0.3 |
| *rad27-p* | 2 ± 1 |
| *rad27Δ* | 604 ± 309 |
| *rad27-K325** | 810 ± 164 |
| *rad27-A358** | 22 ± 1 |
| *rad27-pX8* | 272 ± 21 |

Mutation to 5-FOA resistance for cells harboring pSH44 grown in omission media is shown.

[a] SD, standard deviation.

[b] All the *rad27* mutants, apart from *rad27-p*, have mutation rates significantly higher ($P < 0.05$) than the wild-type rate.

*cdc9-p*. The temperature-sensitive lethality of *rad27-pX8* and the slow growth phenotype of *rad27-A358** with *cdc9-p* are another indication of the balance that must be achieved among the flap endonuclease and DNA ligase I in binding to PCNA.

**Mutation to canavanine resistance and repeat tract instability:** As part of our initial characterization of the *rad27* alleles, we determined the rate of spontaneous forward mutation to canavanine resistance. The rate of mutagenesis is significantly higher in all the *rad27* alleles tested, apart from *rad27-p*, when compared to the wild type ($P < 0.05$) (Table 2). The *rad27Δ* and *rad27-K325** alleles confer mutation rates >100-fold that of the wild type. The other two *rad27* alleles, *rad27-A358** and *rad27-pX8*, have 3-fold and 17-fold higher mutation rates than that of the wild type, respectively. These results show the relative importance of the C-terminal domains of the flap endonuclease in mutation avoidance. In particular, the mutation rate of the *rad27-pX8* mutant shows the necessity of a complete PIP box in mutation avoidance. The mutation rate of *rad27-K325** is greater than the product of the mutation rates of *rad27-A358** and *rad27-pX8*. The *rad27-K325** mutation eliminates the PIP box and all residues C-terminal to it. In particular, it eliminates a set of residues C-terminal to the PIP box that are retained by the *rad27-A358** mutation. These residues contact the IDCL of PCNA and their elimination in *rad27-K325** may account for the mutant's synergistic mutation rate.

We also made another test of mutation avoidance, using a standard assay for dinucleotide repeat instability. We introduced a plasmid containing a run of GT repeats embedded in frame in *URA3* into the *rad27* mutants (HENDERSON and PETES 1992). As in the canavanine resistance assay, the *rad27-K325** mutation confers a mutation rate to Ura− that is as great, if not greater, than *rad27Δ* (Table 3). The *rad27-pX8* and *rad27-A358** mutations confer rates proportional to their mutation

rate to canavanine resistance. While the product of the mutation rates to Ura− conferred by these two mutations is greater than the rate found for the *rad27-K325** mutation, the sum of the two is less than the *rad27-K325** rate, again suggesting that the extra portion of the flap endonuclease between residues 347 and 358 is important for mutation avoidance.

**Characterization of *rad27* alleles in combination with *exo1Δ*, *rad2Δ*, and *rad51Δ*:** The involvement of the flap endonuclease in multiple DNA transactions is evidenced by the genetic interaction of *rad27Δ* with mutations of the repair and recombination genes *EXO1*, *RAD2*, and *RAD51*. To characterize and to distinguish among our *rad27* alleles, we mated them to *exo1Δ*, *rad2Δ*, and *rad51Δ*. We sporulated the resulting diploids and germinated the spores at 25° on YPD. If the spores germinated, we then tested their growth at 35° (supporting information, Table S1).

*EXO1* encodes exonuclease I, a 5′–3′ exonuclease (TISHKOFF *et al.* 1997a). *rad27Δ* is synthetically lethal in combination with *exo1Δ* (TISHKOFF *et al.* 1997a). This lethality is thought to occur because Exo1 has 5′-flap cleavage capability that is redundant with the flap endonuclease activity of the enzyme (TRAN *et al.* 2002). In addition, high-level expression of the nucleases encoded by *EXO1* suppresses the hyper-mutation phenotype of *rad27Δ* (SUN *et al.* 2003). Like the *rad27Δ exo1Δ* double mutant, the *rad27-K325** *exo1Δ* double mutant is inviable (Table S1). *rad27-pX8 exo1Δ* is viable at 25° but exhibits a temperature-sensitive phenotype at 35°. *rad27-A358**, the mutant with the least severe phenotype, is viable in combination with *exo1Δ* at both temperatures. These data show that the *rad27Δ* and *rad27-K325** mutants, which are temperature sensitive and have high mutation rates, have an absolute requirement for *EXO1*. The *EXO1* requirement for growth of *rad27-pX8* at 35° shows the need for this nuclease to support viability of the flap

endonuclease mutant lacking all the residues of its PIP box.

*RAD2* encodes an endonuclease homologous to the flap endonuclease and the *EXO1* encoded nuclease that is required for nucleotide excision repair. In contrast to the inviability of the *exo1Δ rad27Δ* double mutant, the *rad2Δ rad27Δ* double mutant is viable, indicating that other nucleases can support DNA replication and repair (REAGAN *et al.* 1995; SOMMERS *et al.* 1995). Like the suppression of *rad27Δ* by high-level expression of *EXO1*, so too can high-level expression of *RAD2* suppress the mutation avoidance phenotype of *rad27Δ* (SUN *et al.* 2003). When we combined *rad2Δ* with our *rad27* alleles, all the double mutants are viable at 25°. However, at 35°, the *rad2Δ* double mutants with *rad27Δ* and *rad27-K325\** are temperature sensitive. The double mutants of the less severe mutations, *rad27-pX8* and *rad27-A358\**, are viable at 35° (Table S1). Because both *rad27Δ* and *rad27-325\** are temperature sensitive as single mutants, the inviability of the *rad2Δ* double mutants does not yield information on the contribution of *RAD2* to their inviability.

*RAD51* encodes the strand exchange protein that facilitates double-strand break repair. Previous studies have shown that the lesions caused by the absence of the flap endonuclease are repaired via the recombinational repair pathway (SYMINGTON 1998). When we attempted to make double mutants of each of our *rad27* alleles with *rad51Δ*, we found that, with the exception of the two alleles with the least severe phenotypes, *rad27-p* and *rad27-A358\**, all are inviable at 25° (Table S1 and REFSLAND and LIVINGSTON 2005). The *rad27-A358\* rad51Δ* double mutant is temperature sensitive at 35° (Table S1). This temperature-induced lethality is interesting because neither the *rad51Δ* nor the *rad27-A358\** single mutant is temperature sensitive. Furthermore, the *rad27-A358\** mutant exhibits slight sensitivity to MMS and has a slightly elevated mutation rate. The synthetic lethality of the *rad27-A358\* rad51Δ* double mutant at 35° suggests that the DSB repair pathway is essential to repair the few lesions generated in the *rad27-A358\** mutant.

**MMS sensitivity of *rad27* mutants:** We also examined the sensitivity of the *rad27-K325\** and *rad27-pX8* mutants to two tests of MMS sensitivity, one at relatively low concentrations in agar that reports base excision repair and one at a high concentration where the chemical acts as a radiomimetic agent. *rad27-K325\** has a sensitivity much like *rad27Δ* at concentrations of MMS of 0.0175 and 0.035% (Figure 4). The *rad27-pX8* mutant is partially resistant at the lower of these two concentrations (Figure 4). The sensitivity can be suppressed by high-copy expression of *EXO1* (Figure 4A) and to a lesser extent by high-copy expression of *RAD2* (Figure 4B).

We also incubated mutants in a higher concentration of MMS that produces double-strand breaks in the DNA. All of the *rad27* mutants are susceptible to DNA damage
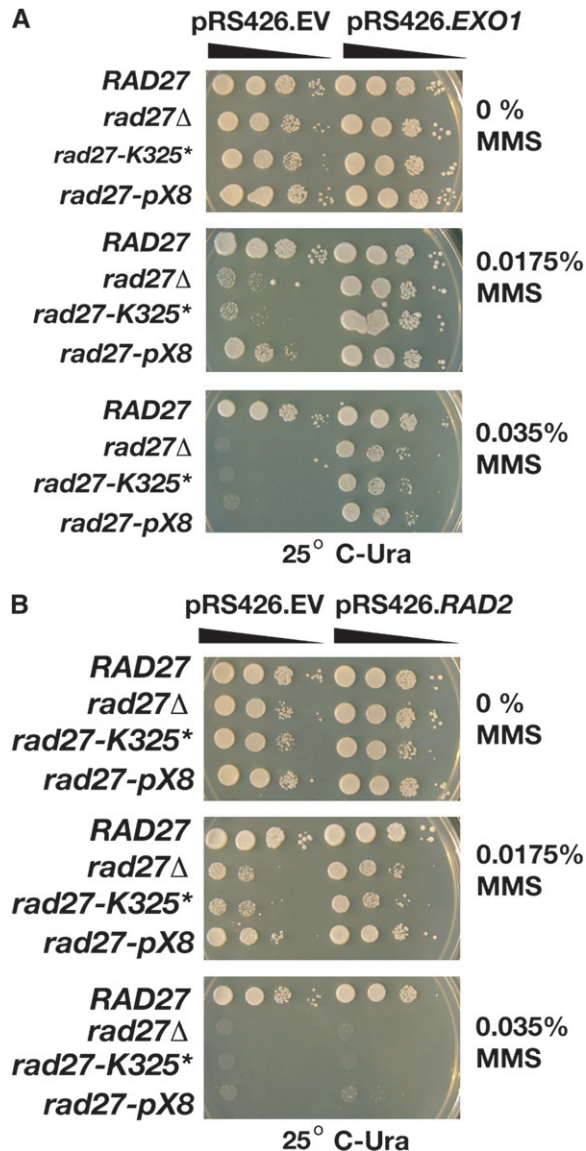


FIGURE 4.—Growth of *rad27* mutants on MMS agar. The mutants were spotted on selective agar containing MMS at 25°. (A) Strains were transformed with either pRS426 (EV empty vector) or pRS426.*EXO1*. (B) Strains were transformed with either pRS246 (EV empty vector) or pRS426.*RAD2*.

induced by this concentration of MMS (Figure 5). The mutants exhibit a gradient in their sensitivity. *rad27-p* is nearly as resistant to MMS as the wild-type parent as previously observed (GARY *et al.* 1999; REFSLAND and LIVINGSTON 2005). *rad27-A358\** is sensitive but more resistant than either *rad27-pX8* or *rad27-K325\**. Both the *rad27-pX8* and *rad27-K325\** mutants retain partial resistance. In the case of the *rad27-pX8* mutant, the decrease in viability describes a biphasic curve that was observed for both isolates that we examined (data not shown). Of greater interest to us is the partial resistance of the *rad27-K325\** mutant in comparison to the null mutant *rad27Δ*. This is an indication that the truncated protein product of the *rad27-K325\** allele retains partial biological function.
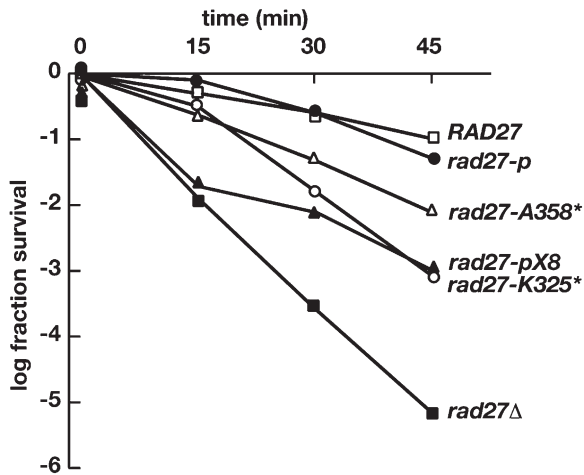
FIGURE 5.—Survival of *rad27* mutants to exposure to 0.5% MMS. Multiple survival studies were carried out for the strains contemporaneously, and the results were averaged. For clarity, the error for each time point is not shown.
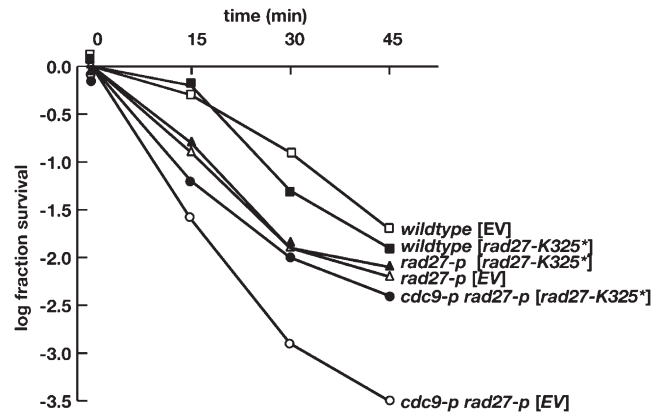


FIGURE 6.—Survival of the *cdc9-p rad27-p* double mutant to exposure to 0.5% MMS. Multiple survival studies were carried out for the strains contemporaneously, and the results were averaged. For clarity, the error for each time point is not shown. For these studies, the strains were grown in selective media, exposed to MMS, and plated on selective agar at 25°.

**High-level expression of *rad27-K325\** suppresses the temperature and MMS sensitivity phenotypes of the *cdc9-p rad27-p* mutant:** While the temperature sensitivity of its growth on YPD, its synthetic lethality in combination with *cdc9-p*, *exo1Δ*, *rad2Δ*, and *rad51Δ*, and its mutation rate do not distinguish the *rad27-K325\** mutant from the null, its partial resistance to MMS suggests that the mutant protein retains partial biological function. The truncated enzyme retains its nuclease domain, and other studies have shown that mutation of its PCNA binding motifs does not destroy its nuclease activity (FRANK *et al.* 2001).

Another indication of the biological activity remaining in the *rad27-K325\** mutant is our observation that this mutant is capable of growth at 35° when plated on selective media (C−Ura) (Figure S1). Its ability to grow on selective media contrasts it to the null, which is unable to grow at on these media at 35°. In addition, when *rad27-K325\** is expressed in the *rad27Δ* strain, it confers growth (Figure S1). Thus, the *rad27-K325\** mutant enzyme retains partial function.

We have also measured whether the protein product of *rad27-K325\** is as abundant as the wild-type protein. Using immunoblotting to detect epitope-tagged proteins, we observed that both the wild-type and the mutant proteins are expressed at similar levels (Figure S2). Thus, partial activities that are retained by the *rad27-K325\** mutant are not likely the result of an unstable protein.

Finally, we transformed the *rad27-K325\** allele expressed from a high-copy plasmid into a strain with partial resistance to MMS to ask whether it could enhance or reduce the partial resistance. The strain we chose was the *cdc9-p rad27-p* double mutant that we had already characterized to have partial MMS resistance (REFSLAND and LIVINGSTON 2005). We tested the

transformed strain for temperature and MMS sensitivity. The *cdc9-p rad27-p* double mutant grows weakly at 35°, and high-level expression of *rad27-K325\** suppresses the temperature-sensitive growth defect although the growth is not as robust as that of the wild type (Figure S3). In addition, high-level expression of *rad27-K325\** increases the MMS resistance of the double mutant nearly to wild-type resistance (Figure 6). These results suggest that the *rad27-K325\** enzyme, despite having its entire C terminus truncated, retains partial biological function. Furthermore, while the product of *rad27-K325\** might have interfered with the weakened binding of the *rad27-p* product to reduce the MMS resistance of the double mutant, it did not do so. While we cannot rule out that a physical interaction between the products of *rad27-K325\** and *rad27-p* accounts for the increase in function, the most straightforward conclusion is that the *rad27-K325\** enzyme retains partial activity.

**High-level expression of PCNA suppresses the temperature and hypermutagenic phenotypes of *rad27-K325\** and *rad27Δ* mutants:** Next, we revisited our initial observation that high-copy expression of *POL30* (PCNA) partially suppresses the temperature-sensitive growth of the *rad27-K325\** mutant on YPD. As our initial observation was done using the mutagenized strain from which we identified the *rad27-K325\** mutation, we transformed the reconstituted *rad27-K325\** mutant with a high-copy plasmid expressing *POL30* and assayed for growth at 25° and 35°. On YPD agar, overexpression of *POL30* partially suppresses the temperature-sensitive growth defect of the *rad27-K325\** mutant (Figure 7, A and B). When the analyses were conducted on selective media, we observed that neither the null mutant nor the *rad27-K325\** mutant that had been transformed with the empty vector exhibited as profound a temperature growth deficit as they did on
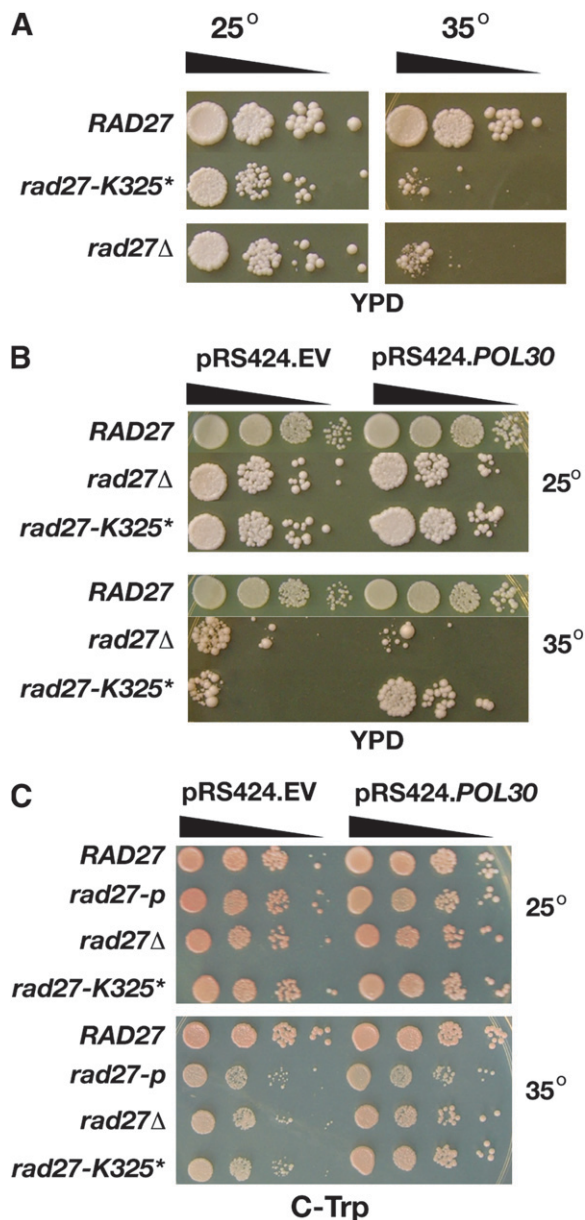
FIGURE 7.—PCNA suppression of *rad27* mutant growth defects on YPD and selective agar. (A) Temperature sensitive growth on YPD agar. (B) YPD agar with pRS424 or pRS424.*POL30*. (C) Selective agar with pRS424 or pRS424.*POL30*.

**TABLE 4**

**Mutation rates in *rad27* mutants**

| Strain | Mutation rate ($\times 10^{-7}$ events/cell/generation) $\pm$ SD[a] | |
|---|---|---|
| | Empty vector[b] | *POL30*[b] |
| *RAD27* | 1.8 ± .3 (1) | 2.3 ± 1 (1) |
| *rad27Δ* | 257 ± 65 (143) | 31 ± 4 (13) |
| *rad27-K325\** | 134 ± 22 (74) | 36 ± 9 (16) |
| *rad27-pX8* | 62 ± 14 (35) | 12 ± 3 (5) |
| *rad27Δ[c]* | 200 ± 38 | 49 ± 20 |

Mutation to canavanine resistance for cells grown in omission media.

[a] SD, standard deviation.

[b] Values normalized to the *RAD27* rates for empty vector (pRS424) or for *POL30*, as appropriate, are shown in parentheses. For all strains except *RAD27*, the pRS424.*POL30* rate is significantly lower ($P < 0.05$) than the empty vector rate.

[c] Strain SSL938.

grown at 36° in liquid culture. In the case of the null allele, we did not measure a reduction in doubling time when PCNA was overexpressed. The results show that suppression of *rad27-K325\** by high-level expression of PCNA is more pronounced than suppression of *rad27Δ*. Thus, while high-level expression of PCNA ameliorates the growth phenotypes of both *rad27-K325\** and *rad27Δ*, the differences in the degree of suppression suggest that more than one mechanism of suppression may be operating in the *POL30* suppression of *rad27-K235\**.

We next tested whether high-copy expression of *POL30* can suppress the canavanine mutation rate of the *rad27* alleles. We found that overexpressing PCNA significantly ($P < 0.05$) decreases the rates of spontaneous mutation to canavanine resistance in all the *rad27* mutants tested, including the null (Table 4). (*rad27-A358\** and *rad27-p* were not tested because they have a low rate of mutagenesis.) Overexpression of PCNA reduces the spontaneous mutation rate of canavanine resistance fourfold in the *rad27-K325\** mutant. Similarly, overexpression of PCNA reduces the spontaneous mutation rate fivefold in the *rad27-pX8* mutant.

In accord with the suppressive effects of PCNA on colony growth, high levels of PCNA also significantly reduce the mutation rate in the null mutant (Table 4). To exclude the possibility that this PCNA suppression results from complementation of a hypomorphic *pol30* mutation in our parental strain, we recovered and sequenced the endogenous *POL30* gene, including its upstream region. Sequencing showed that our parental strain does not harbor any *pol30* mutations. As further assurance, we repeated this study in a *rad27Δ* strain derived from a different strain background. The suppressive effect of high-level expression of PCNA on canavanine resistance was also seen in strain SSL938, a *rad27Δ* strain that is not isogenic to our *rad27* alleles (Table 4). The ability of PCNA to suppress both

YPD agar at 35° (Figure 7C). (We note the variation in growth of *rad27* mutants on rich and synthetic media in Figure 7 and previous figures. This variation among different media is reproducible.) When transformed with the plasmid that expresses PCNA, each mutant grows better as seen by the larger colonies that form (Figure 7C). Thus, a high level of PCNA has a suppressive effect on both mutations.

In an attempt to provide a quantitative demonstration of the suppressive effects of *POL30* on the two alleles, we measured doubling times. High-level expression of *POL30* reduced significantly ($P < 0.05$) the doubling time of two *rad27-K325\** isolates by 51 and 22 min when

**Mutation rates in *exo1* and *rad27-pX8* mutants**

| Strain | Mutation rate ($\times 10^{-7}$ events/cell/generation) $\pm$ SD[a] | |
| | Empty vector[b] | POL30[b] |
| --- | --- | --- |
| *RAD27* | 2.8 ± 0.7 (1) | 5.8 ± 3 (1) |
| *exo1Δ* | 12 ± 3 (4) | 11 ± 1 (2) |
| *rad27-pX8* | 53 ± 7 (19) | 12 ± 3 (2) |
| *exo1Δ rad27-pX8* | 289 ± 59 (103) | 99 ± 26 (17) |

Mutation to canavanine resistance for cells grown in omission media is shown.

[a] SD, standard deviation.

[b] Values normalized to the *RAD27* rates for empty vector (pRS424) or for *POL30*, as appropriate, are shown in parentheses. The pRS424.*POL30* rate is significantly lower ($P < 0.05$) than the empty vector rate for the *rad27-pX8* and the *exo1Δ rad27-pX8* mutants.

hypomorphic and null *rad27* alleles suggests that additional PCNA helps mutant cells by increasing flap endonuclease function in the hypomorphic mutants, *rad27-K325** and *rad27-pX8*, or by providing an opportunity for another nuclease to take the place of the flap endonuclease or both.

We have also have tested whether high-copy expression of PCNA could suppress in other assays of mutation avoidance and DNA repair. High-copy expression of PCNA suppresses the incidence of GT repeat tract instability (Table S2). (Note that the plasmid expressing *POL30* in the repeat tract assay carried a different nutritional selection marker from that in the other assay, *LEU2* instead of *TRP1*. Thus, the suppressive response is not likely influenced by nutritional selection.) We did not find suppression of MMS sensitivity either on agar or in liquid culture (data not shown). The suppression recorded in some assays and not others may indicate differences in the protein and enzyme requirements in each assay and in their respective interactions with PCNA.

The high-copy suppression of *rad27-pX8* afforded us the opportunity to examine the behavior of the *rad27-pX8 exo1Δ* double mutant. Both mutations have a mild mutator phenotype in comparison to *rad27Δ*. When combined, they produce a synergistic mutation rate that is noteworthy because of the low mutation rate of the *exo1Δ* mutant (Table 5). This genetic interaction is consistent with the temperature lethality of the double mutant although the mutation rates were determined at the permissive temperature (25°). Next, we introduced *POL30* into these strains to determine the extent of suppression. High-copy expression of *POL30* has previously been reported to suppress *exo1Δ* although the results show very slight suppression (Amin *et al.* 2001). Because the mutation rate to canavanine resistance is only slightly elevated in our *exo1Δ* mutant over the wild-

type rate, we were unable to detect *POL30* suppression. *POL30* is able to suppress the *rad27-pX8 exo1Δ* double mutant, but it is unable to bring it down to the level of the most severe single mutant *rad27-pX8* (Table 5). The results suggest that the synergism underlies a necessity for each enzyme to act on the other's deficiency and that the suppression by *POL30* cannot make up for this necessity.

**High-level expression of PCNA does not suppress the temperature-sensitive phenotypes of the DNA ligase I mutant *cdc9-1* or the helicase–endonuclease mutant *dna2-1*:** To examine whether suppression by high-level expression of PCNA is specific to *rad27* mutations, we tested two additional replication mutations. We chose mutations of the enzymes most closely associated with the activity of the flap endonuclease in DNA replication. During DNA replication, the flap endonuclease is thought to bring the *DNA2* helicase–nuclease to the replication fork because the helicase–nuclease binds to the flap endonuclease (Budd and Campbell 1997; Stewart *et al.* 2006). However, PCNA is not known to bind to the *DNA2*-encoded helicase–nuclease. The *DNA2* nuclease may participate in primer removal along with the flap endonuclease (Bae *et al.* 1998, 2002; Budd *et al.* 2000; Stewart *et al.* 2006). The role of the *DNA2* helicase activity remains enigmatic, although it, along with the nuclease activity, is essential to the yeast cell (Budd *et al.* 1995, 2000). High-level expression of *DNA2* suppresses *rad27Δ* (Budd and Campbell 1997). For our studies on PCNA suppression we chose to test the *dna2-1* mutant that has an alteration in the enzyme's helicase domain and is temperature sensitive for growth (Budd and Campbell 1995). This *dna2* mutation is synthetically lethal with *rad27Δ* (Budd and Campbell 1997). We also tested the DNA ligase I mutation *cdc9-1* that is temperature sensitive for growth.

Each mutant was transformed with a high-copy plasmid harboring *POL30* and grown at 25° and 35°. High-copy expression of *POL30* does not suppress temperature-sensitive lethality of either mutant (data not shown). Comparisons among temperature-sensitive mutations of multiple genes are difficult because of potential differences in phenotypic characteristics. Nevertheless, these results show that the suppressive effect of PCNA is specific to the flap endonuclease mutants rather than a generic, ameliorating effect that high levels of PCNA might have on other DNA replication proteins.

***rad27-K325** is synthetically lethal with *pol30-79* and *pol30-90*:** Finally, because our mutations have altered the PCNA binding capacity of the flap endonuclease, we were interested in whether they could be combined with *pol30* mutations that are known to affect the flap endonuclease. Mutational analysis of *POL30* has identified two mutations of PCNA that have profound effects on flap endonuclease binding and activation (Eissenberg *et al.* 1997). *pol30-79* (L126A/I128A) and

*pol30-90* (P252A/K253A) are mutations in the hydrophobic pocket of the IDCL and the C-terminal end of PCNA, respectively. *In vitro*, the *pol30-79* mutant protein fails to interact with the flap endonuclease but manages to stimulate the nuclease activity. In contrast, the *pol30-90* mutant protein binds to the flap endonuclease but fails to stimulate its nuclease activity (GOMES and BURGERS 2000). In genetic studies, EISSENBERG *et al.* (1997) reported that the *pol30-79 rad27Δ* and *pol30-90 rad27Δ* double mutants are viable but exhibit poor growth at 23°.

To determine whether our *rad27* alleles are viable in combination with these PCNA mutations, we mated *pol30-79* and *pol30-90* with the *rad27* alleles. The diploids were sporulated and dissected onto YPD at 25°. In our strain background both *rad27Δ* and *rad27-K325** proved inviable with *pol30-79*, while the *rad27-A358* pol30-79* and *rad27-pX8 pol30-79* double mutants were viable but grew slower than either single mutant (data not shown). More interesting to us was the observation that the *rad27Δ pol30-90* double mutant is viable and grows slowly at 25° [as previously observed (EISSENBERG *et al.* 1997)], whereas the combination of *rad27-K325** with *pol30-90* is inviable (Figure 8A). We were able to streak out the *rad27Δ pol30-90* spore colonies onto rich media where they formed microcolonies after 10 days at 25° (Figure 8B). As expected, the combinations of *rad27-A358** and *rad27-pX8* with *pol30-90* yielded viable spores. When grown on rich media, these double mutants exhibit growth defects that are commensurate with the severity of their mutator and repair phenotypes (Figure 8B). For example, *pol30-90 rad27-pX8* grows slower than does *pol30-90 rad27-A358**. Thus, *rad27-K325** is anomalous in that it exhibits synthetic lethality with *pol30-90* that is not shown by the null mutation.

## DISCUSSION

PCNA is responsible for bringing the flap endonuclease and DNA ligase I into lagging-strand synthesis during DNA replication. How PCNA orders these final steps in lagging-strand synthesis is not well understood. The recovery of a C-terminal truncated mutation of the flap endonuclease missing its PCNA-interacting domains that is synthetically lethal with a mutation in DNA ligase I that weakens its interaction with PCNA contributes to our understanding of how the interactions among PCNA, the flap endonuclease, and DNA ligase I occur. The recovery of the *rad27-K325** allele in a synthetic lethal screen with an allele of DNA ligase I, *cdc9-p*, and the mutant's genetic interactions with *pol30-90* might not have been unexpected. Prior studies have shown a genetic interaction between mutations of the flap endonuclease and DNA ligase that weaken each enzyme's binding to PCNA, *rad27-p* and *cdc9-p* (REFSLAND and LIVINGSTON 2005), and between *rad27Δ* and various *pol30* alleles (EISSENBERG *et al.* 1997).
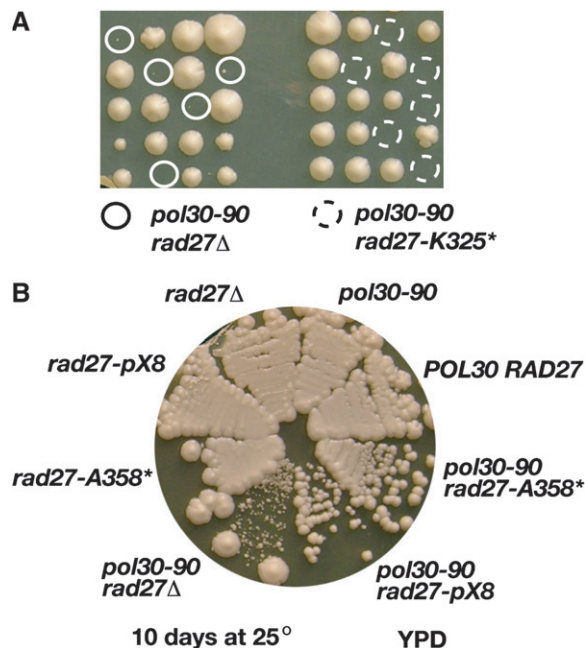


FIGURE 8.—Interaction between *rad27* mutations and the *pol30-90* mutation. (A) Germination of spore colonies that have the genotype *rad27Δ pol30-90* or *rad27-K325* pol30-90*. (B) The difference in colony size among the *rad27 pol30-90* double mutants after 10 days of incubation at 25°.

The *rad27* allele we recovered from our screen, *rad27-K325**, and the ones we created, *rad27-A358** and *rad27-pX8*, have a spectrum of phenotypes that are consistent among most of the tests of growth, mutation avoidance, and DNA repair. In general, they fall within a gradient that extends from the null mutant with the most severe display of a phenotype to the wild type. In between, the *rad27-K325** mutant is closest to the null mutant and the *rad27-A358** mutant and the *rad27-p* mutant are closest to the wild type in their phenotypes. The *rad27-pX8* mutant occupies a middle ground. The combinations of these *rad27* alleles with *rad2Δ*, exo1Δ, *cdc9-p*, and *rad51Δ* exemplify a novel example of this gradient. While all *rad27* mutations are viable in combination with *rad2Δ* at 25°, the most severe ones, *rad27Δ* and *rad27-K325**, are temperature sensitive for growth, while the two others, *rad27-pX8* and *rad27-A358**, are not. This again suggests that *rad2Δ* does not exacerbate the mutant phenotypes of *rad27* mutations. Next, while the most severe mutations are inviable when combined with exo1Δ, the *rad27-pX8* double mutant is temperature sensitive for growth and the *rad27-A358** mutant grows at the restrictive temperature. When the alleles are combined with *cdc9-p*, not only does the *rad27-pX8* double mutant become inviable at 35° but also the *rad27-A358** double mutant exhibits a growth defect at that temperature. Finally, while all mutations except *rad27-A358** are inviable in combination with *rad51Δ*, this weakest mutation becomes temperature sensitive. The consistency among tests suggests that progressive loss or mutational sub-

stitution in the C-terminal tail plays nearly evenly among the myriad of functions that the flap endonuclease fulfills in the cell.

While the *rad27* mutations fall into a pattern, each has properties that are unique. First, the *rad27-K325\** mutant, while it shares a number of phenotypes with *rad27Δ*, is different from *rad27Δ*. Like *rad27Δ*, *rad27-K325\** is lethal in combination with *cdc9-p*, confers temperature-sensitive growth, and is as mutagenic as *rad27Δ*. Similarly, both are partially suppressible by high-level expression of PCNA. Furthermore, when we combined *rad27Δ* and *rad27-K325\** with mutations of genes required for DNA repair, *exo1Δ* and *rad2Δ*, and recombination, *rad51Δ*, they proved to behave similarly. Both are synthetically lethal when combined with *exo1Δ* and *rad51Δ* and are temperature sensitive with *rad2Δ*.

*rad27-K325\** can be distinguished from *rad27Δ* in tests that show that the truncated protein is likely to retain some biological activity and in other tests that show that the mutant protein may confer a degree of toxicity to the cell. First, the *rad27-K325\** mutant is more resistant to MMS exposure than is the null mutant. The partial resistance suggests that the mutant protein is partially capable in the repair of the damage caused by the radiomimetic agent. Retention of partial activity might be expected because the mutant retains all the nuclease domains of the enzyme and the mutant protein appears as stable as the wild-type protein. Retention of partial activity in one assay (MMS resistance) and not in another (mutation avoidance) is likely a reflection of the multiple activities that the enzyme possesses. Depending on the substrate presented to the purified enzyme, it acts as a flap endonuclease, as an exonuclease, or as a gap endonuclease (Zheng *et al.* 2005; Singh *et al.* 2007). Furthermore, flap endonuclease mutations differentially affect the multiple activities (Xie *et al.* 2001; Zheng *et al.* 2005; Singh *et al.* 2007). Thus, the difference in phenotypic penetrance conferred by *rad27-K325\** may reflect the possibility that the mutant protein can act on some chromosomal substrates but not on others.

We have found that while high-level expression of *rad27-K325\** did not increase the level of MMS resistance of the null mutant beyond the level seen in the *rad27-K325\** strain (data not shown), high-level expression of *rad27-K325\** increases the resistance of the *cdc9-p rad27-p* double mutant to MMS exposure. Why the product of the *rad27-K325\** allele increases the MMS resistance of the *cdc9-p rad27-p* double mutant, where the PIP box of both enzymes has been mutated, is not obvious. Such results might be accounted for by physical interactions between the two mutated types of the flap endonuclease or by a partition of function between the two mutant types. The first explanation does not appear to be plausible because the flap endonuclease is not known to form multimers, the product of the *rad27-K325\** allele appears to be stable, and the product of the *rad27-p* allele is presumed to be stable by virtue of its

near wild-type MMS resistance. The second explanation of a partition of function cannot be ruled out, but what function one mutant form could confer to the other is not apparent. We suggest that complementation may result from the manner in which the two mutant forms of the flap endonuclease bind to PCNA. Binding studies show that removal of the PIP box, as created in the *rad27-K325\** allele, surprisingly increases the affinity of the flap endonuclease for PCNA (Guo *et al.* 2008). Possibly, in the suppressed double mutant, the product of *rad27-K325\** binds first to PCNA and alters the conformation of PCNA so that the product of *rad27-p* is able to bind in an active conformation by a mechanism first suggested by Gomes and Burgers (2000).

The other assay that shows the difference between the *rad27-K325\** allele and the null allele is the lethality that the former has with *pol30-90*. While *rad27Δ pol30-90* grows slowly, it is not lethal as is *rad27-K325\* pol30-90*. This not only shows a difference between the two alleles but also shows that *rad27-K325\** may confer a slight toxicity to the cell when PCNA is mutated. We also note that in measures of mutation avoidance, the *rad27-K325\** mutant exhibits slightly greater promiscuity than the null mutant (Tables 2 and 3). Toxicity is not unprecedented, as a *rad27* allele, *rad27-n*, with a mutation of the nuclease catalytic site is toxic to the yeast cell. This deleterious allele is detoxified by mutating the PIP box (Gary *et al.* 1999). Thus, some mutant forms of the flap endonuclease appear to bind to PCNA in a manner that inhibits the binding of other enzymes, in turn inhibiting DNA replication.

The *rad27-pX8* mutation that substitutes α-helical forming residues for the eight amino acids of the PIP box is interesting because of the severity of its phenotypes in comparison to *rad27-p*, a mutation of two of the eight PIP box residues. It is distinctly more sensitive to MMS than *rad27-p* and lethal in combination *rad51Δ*. These phenotypes suggest a strong need for interaction between the flap endonuclease and PCNA in the incidence of DSBs or in recovery from DSBs. Furthermore, it becomes temperature sensitive when combined with *exo1Δ*. In the case of the *exo1Δ rad27-pX8* double mutant, we note that its synergistic mutation rate at 25° places this *rad27* mutation in a class of mutations uncovered in a screen for enhancers of the weak *exo1Δ* mutator phenotype (Amin *et al.* 2001). The simplest explanation for the temperature sensitivity of the *exo1Δ rad27-pX8* double mutant is that the mutant enzyme becomes inactive, and the synthetic lethality reflects the synthetic lethality of the *exo1Δ rad27Δ* double mutant. Considering that the *rad27-pX8* mutant is viable at 35° and, consequently, likely to retain partial function, the synthetic lethality may reflect the sensitivity of the *exo1Δ* mutant to diminished *RAD27* function.

The *rad27-A358\** mutant is interesting because of its seemingly mild phenotypes in combination with its absolute requirement for *CDC9* and *RAD51* at 35°. In

particular, its requirement for *RAD51* implies that the mutant protein introduces DNA lesions that have an absolute requirement for the DSB repair system.

One result that comments strongly on the role of PCNA in mediating the events of DNA replication and repair is that overexpression of PCNA reduces the rates of spontaneous mutations not only in the two partial hypomorphic mutants *rad27-K325** and *rad27-pX8* but also in the null mutant. A possible explanation for suppression of *rad27Δ* is that the large quantity of PCNA recruits a novel nuclease that suppresses the mutant phenotype. Potential candidates for suppression of the null are the nucleases encoded by *EXO1* and *RAD2* and the nuclease activity of the *DNA2* helicase–nuclease. High levels of Exo1 compensate for the loss of the flap endonuclease in *rad27Δ* mutants by suppressing the temperature-induced lethality and mutation avoidance phenotypes (TISHKOFF *et al.* 1997a; SUN *et al.* 2003). High-level expression of *RAD2* also compensates (SUN *et al.* 2003). While both Rad2 family members suppress the temperature-sensitive growth and mutator activity of *rad27Δ*, only *RAD2* high-level expression suppresses its MMS sensitivity (SUN *et al.* 2003). In addition, high-level expression of *DNA2* partially compensates for the absence of the flap endonuclease (BUDD and CAMPBELL 1997). While all three activities might compensate, we note that none of the three have been shown to directly interact with PCNA.

Suppression of *rad27-K325** and *rad27-pX8* by overexpression of PCNA may occur by a second mechanism that does not solely involve compensation by another nuclease. Instead, suppression may occur, either in part or in whole, by increased binding of the mutant nucleases to PCNA by mass action. In discussing this possibility, we take into account the many contacts that the human flap endonuclease ortholog makes with PCNA and the mechanism by which the nuclease is thought to reach DNA substrates. Co-crystallization of human FEN-1 with PCNA revealed that the flap endonuclease makes contacts throughout its length, including the core nuclease domain that remains in our *rad27-K325** mutant (SAKURAI *et al.* 2005). The multiple contacts also include segments of FEN-1 that extend on either side its PIP box. In addition, a truncated mutant of the human flap endonuclease missing its PIP box is capable of binding to PCNA (GUO *et al.* 2008). Thus, all mutant forms that we have created should be capable of binding to PCNA with reduced affinity. Increasing the amount of PCNA should increase the amount of binding, and that may be partially responsible for suppression of the mutant phenotypes.

The activity of the flap endonuclease is greatly increased by its binding to PCNA (LI *et al.* 1995; JONSSON *et al.* 1998; GOMES and BURGERS 2000; TOM *et al.* 2000; FRANK *et al.* 2001). The mechanism that accounts for this activation suggests that the enzyme first binds to PCNA through its PIP box in a manner that precludes its binding to DNA substrates and then rotates through a hinge region between its core and the PIP box to allow the enzyme to meet its substrates (GOMES and BURGERS 2000; SAKURAI *et al.* 2005). This two-step mechanism was first proposed by the difference between the binding affinity and nuclease activation of the *pol30-79* and *pol30-90* mutant copies of PCNA (GOMES and BURGERS 2000). The *pol30-79* mutation lies in the hydrophobic pocket of the IDCL and interacts directly with the PIP box, while the *pol30-90* mutation is adjacent to the hinge domain and may be important for rotation. The *pol30-79* mutant protein does not bind strongly to the flap endonuclease but can still stimulate its activity, while the *pol30-90* mutant protein binds more strongly but does not stimulate the nuclease activity. The two modes of flap endonuclease binding to PCNA are supported by the co-crystallization results that demonstrate a hinge permitting rotation of the nuclease across the front face into the PCNA-encircled DNA (SAKURAI *et al.* 2005). The difference in behavior between the two *pol30* mutations suggests that while initial binding is important, it is not necessary for stimulation, while rotation through the hinge is necessary to achieve stimulation.

The behavior of our mutations can be interpreted on the basis of the proposed mechanism. We suggest that the product of *rad27-K325** retains partial biological activity and binds weakly to PCNA. Because it is missing the hinge, it cannot reach most DNA substrates. The extra toxicity of *rad27-K325** when combined with *pol30-90* in comparison to the viability of the *rad27Δ pol30-90* double mutant supports our view that the mutant protein binds to PCNA through it core domain and excludes binding by other PCNA binding proteins. In essence, the product of *rad27-K325** may act as a competitive inhibitor, and this effect is exacerbated in the *pol30-90* mutant that weakens the interaction between PCNA and its other partners. This competitive effect may also operate to prevent the product of *cdc9-p* from successfully binding to PCNA, and this exclusion may lead to the synthetic lethality between *cdc9-p* and *rad27-K325**. On the basis of the rotation model, we interpret our results to suggest that the product of *rad27-pX8* has difficulty with the initial steps of binding to PCNA but possesses the hinge region and C-terminal residues so it is able to rotate and complete catalysis if binding occurs. Combining *rad27-pX8* with *pol30-90* diminishes both the initial binding and possibly the rotation event as the substitutions in *rad27-pX8* change the glutamine residue that interacts with the amino acid residues of PCNA that are mutated in *pol30-90*. These additive changes make the double mutant grow slowly. Finally, the product of the *rad27-A358** mutant possesses all the binding regions and is nearly completely functional. Thus, the behavior of our flap endonuclease alleles is consistent with the binding studies and the proposed rotation model.

Finally, we return to the hypothesis that propelled these studies. On the basis of our studies of CAG repeat tract expansions, we suggested that PCNA mediates the ordered entry of the flap endonuclease and DNA ligase I into replication (Refsland and Livingston 2005). We began this study with a synthetic lethal screen. As befitting a synthetic lethal screen, we used a *cdc9* allele that can best be characterized as having a mild phenotype, particularly as it grows throughout a range of temperatures and does not exhibit a severe sensitivity to MMS exposure that is seen for other *cdc9* mutations (Hartwell *et al.* 1973; Montelone *et al.* 1981; Refsland and Livingston 2005). Our synthetic lethal screen with *cdc9-p* yielded a *rad27* mutation. Considering that *rad27Δ* is synthetically lethal with many yeast genes, especially those involved in DNA transactions, the appearance of a flap endonuclease mutation with seemingly little activity might not have been unexpected (Tong *et al.* 2004; Loeillet *et al.* 2005; Pan *et al.* 2006). While we have not systematically screened the collection of yeast gene deletions or combined *cdc9-p* with a panel of other replication and repair mutations, the finding of a new *rad27* allele might not have been by chance alone. Previously, we found that we could combine *rad27Δ* with *cdc9-1* at a permissive temperature (Ireland *et al.* 2000). Thus, while direct comparisons between *cdc9-1* and *cdc9-p* have not been made, the absolute lethality of *rad27-K325** with *cdc9-p* is novel. The novelty is that the combination of the *cdc9* and *rad27* alleles with the less severe phenotypes (*cdc9-p* and *rad27-K325**) is lethal while the combination of the alleles with more severe phenotypes (*cdc9-1* and *rad27Δ*) is not.

More important, the absolute lethality of *rad27Δ* and *rad27-K325**, the temperature-sensitive lethality of *rad27-pX8*, and the temperature-impaired growth of *rad27-A358** and *rad27-p* when combined with *cdc9-p* suggest a strong cellular requirement that both enzymes need to be able to interact with PCNA to complete DNA replication and repair. Thus, the inability of the double mutants to grow is best described as a synergistic effect. The relationship is clearly not one of epistasis. We argue that the effect is greater than additive, making it synergistic. We might have expected the double mutants to grow weakly if their combination were additive. In support of our contention, we note that the triple mutant of *cdc9-p rad27-p pol30-90* is viable at 30° (Refsland and Livingston 2005), whereas the *cdc9-p rad27-K325** double mutant is not. By this view, the triple mutant shows an additive effect and the *cdc9-p rad27-K325** double mutant is synergistic. While our arguments may be circumstantial, our results warrant further examination of the role PCNA plays in ordering the entry of the flap endonuclease and DNA ligase I into replication and repair.

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# GENETICS

## C-Terminal Flap Endonuclease (*rad27*) Mutations: Lethal Interactions With a DNA Ligase I Mutation (*cdc9-p*) and Suppression by Proliferating Cell Nuclear Antigen (*POL30*) in *Saccharomyces cerevisiae*

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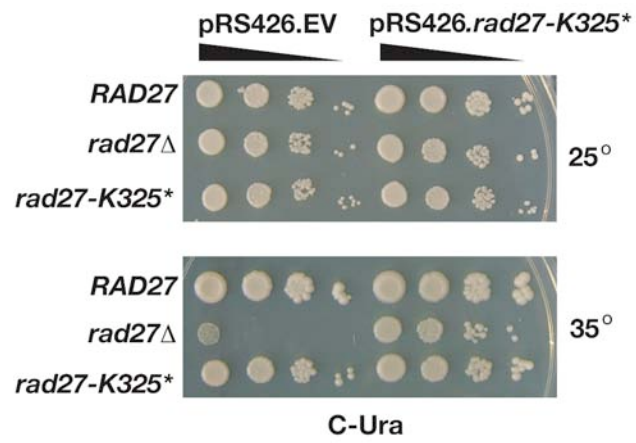
K. K. Karanja and D. M. Livingston



FIGURE S1.—Growth of *rad27Δ* and *rad27-K325\** mutants on selective agar. Dilutions of mutants transformed with either pRS426 or pRS426.*rad27-K325\** were spotted on agar lacking uracil and incubated at 25° and 35°.
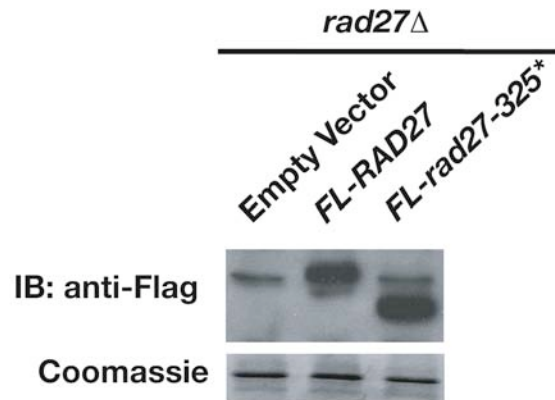
FIGURE S2.— Expression of epitope-tagged proteins. Epitope tagged *RAD27* and *rad27-K325\** were constructed by inserting three flag-epitopes (3X-DYKDDDDK) into the coding sequence such that the tag appeared after the start codon and the gene was under the control of the *RAD27* promoter. The constructs were placed into pRS316.To detect epitope tagged flap endonuclease, SSL533 (*rad27Δ*) was transformed with plasmids harboring flag-tagged *RAD27*, flag-tagged *rad27-K325\**, and an empty vector control. The transformants were grown in selective media to log phase (OD$_{600}$ ~ 0.6 - 1) at 25° and proteins were extracted with trichloroacetic acid. The proteins were subjected to SDS-PAGE, transferred to a nitrocellulose membrane and visualized by immunoblotting with M2 monoclonal anti-Flag antibodies (Sigma-Adrich, St. Louis, MO). The blot shows proteins of the predicted sizes for wild-type and mutant proteins. (A minor nonspecific band appears at the same mobility as full-length product.) A loading control was performed by concurrently running another SDS-PAGE gel, which was used for Coomassie staining.
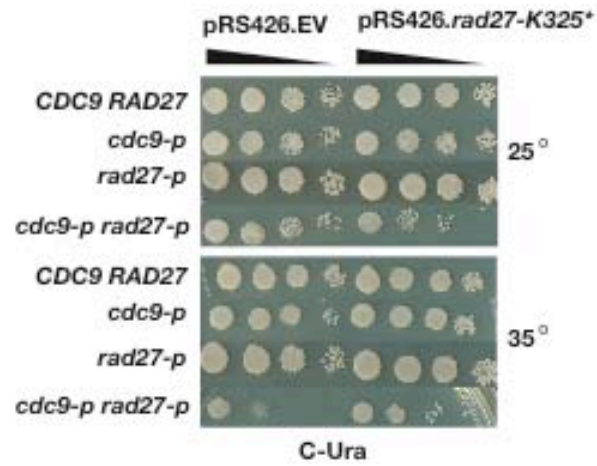
FIGURE S3.— Growth of the *cdc9-p rad27-p* double mutants. Dilutions of mutants transformed with either pRS426 or pRS426.*rad27-K325** were spotted on agar lacking uracil and incubated at 25° and 35°. (Note that the *rad27-p* mutant was spotted on a different agar dish and subjected to identical manipulation.)

**TABLE S1**

**The viability of *rad27* double mutants[a]**

| Genotype | 25° | 35° |
|---|---|---|
| *cdc9-p  rad27-p*[b] | Viable | Viable |
| *cdc9-p  rad27Δ* | Inviable | - |
| *cdc9-p  rad27-K325** | Inviable | - |
| *cdc9-p  rad27-A358** | Viable | Slow Growth |
| *cdc9-p  rad27-pX8* | Viable | Inviable |
| | | |
| *exo1Δ  rad27-p* | Viable | Viable |
| *exo1Δ  rad27Δ* | Inviable | - |
| *exo1Δ  rad27-K325** | Inviable | - |
| *exo1Δ  rad27-A358** | Viable | Viable |
| *exo1Δ  rad27-pX8* | Viable | Inviable |
| | | |
| *rad2Δ  rad27-p* | Viable | Viable |
| *rad2Δ  rad27Δ* | Viable | Inviable |
| *rad2Δ  rad27-K325** | Viable | Inviable |
| *rad2Δ  rad27-A358** | Viable | Viable |
| *rad2Δ  rad27-pX8* | Viable | Viable |
| | | |
| *rad51Δ  rad27-p*[b] | Viable | Viable |
| *rad51Δ  rad27Δ* | Inviable | - |
| *rad51Δ  rad27-K325** | Inviable | - |
| *rad51Δ  rad27-A358** | Viable | Inviable |
| *rad51Δ  rad27-pX8* | Inviable | - |

[a] Haploid parents were mated to produce diploid strains that were sporulated. Spores from tetrads were germinated at 25° and viable spores were subsequently streaked on YPD media and grown at 25° and 35° and viability was assessed after 3 days.

[b] This double mutant had previously been characterized in REFSLAND and LIVINGSTON 2005.

**TABLE S2**

**Mutation rates of a GT repeat tract in *rad27* mutants**

| | Mutation rate (X10⁻⁶ events/cell/generation) ± SD[a] | |
| | --- | --- |
| Strain | Empty vector[b] | *POL30*[b] |
| *RAD27* | 1.0 ± .7 (1) | .29 ± .09 (1) |
| *rad27Δ* | 466 ± 31 (466) | 37 ± 30 (127) |
| *rad27-K325\** | 369 ± 15 (369) | 29 ± 18 (100) |

[a]  SD, standard deviation

[b] Values normalized to the *RAD27* rates for empty vector (pRS425) or for *POL30*, as appropriate, are shown in parentheses. For strains *rad27Δ* and *rad27-K325\**, the pRS425.*POL30* rate is significantly lower ($P<0.05$) than the empty vector rate. Note that the *RAD27* trials were repeated six times without reaching statistical significance.