A Mutation of the Yeast Gene Encoding PCNA Destabilizes Both Microsatellite and Minisatellite DNA Sequences

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ABSTRACT

The *POL30* gene of the yeast *Saccharomyces cerevisiae* encodes the proliferating cell nuclear antigen (PCNA), a protein required for processive DNA synthesis by DNA polymerase δ and ε. We examined the effects of the *pol30-52* mutation on the stability of microsatellite (1- to 8-bp repeat units) and minisatellite (20-bp repeat units) DNA sequences. It had previously been shown that this mutation destabilizes dinucleotide repeats 150-fold and that this effect is primarily due to defects in DNA mismatch repair. From our analysis of the effects of *pol30-52* on classes of repetitive DNA with longer repeat unit lengths, we conclude that this mutation may also elevate the rate of DNA polymerase slippage. The effect of *pol30-52* on tracts of repetitive DNA with large repeat unit lengths was similar, but not identical, to that observed previously for *pol3-t*, a temperature-sensitive mutation affecting DNA polymerase δ. Strains with both *pol30-52* and *pol3-t* mutations grew extremely slowly and had minisatellite mutation rates considerably greater than those observed in either single mutant strain.

ALL eukaryotic genomes contain tracts of simple repetitive DNA, regions in which a small number of bases are tandemly repeated (Tautz and Schlotterer 1994). These repetitive regions can be classified as either microsatellites (repeat units from 1–13 bp in arrays of 10–60 bp) or minisatellites (repeat units >15 bp, often in arrays of several hundred base pairs). In *Escherichia coli* (Levinson and Gutman 1987), *Saccharomyces cerevisiae* (Henderson and Petes 1992), and mammals (Weber and Wong 1993), simple repetitive DNA tracts are unstable, altering in length by additions or deletions of small numbers of repeat units.

On the basis of studies of the genetic control of microsatellite stability in bacteria and yeast (reviewed by Sia *et al.* 1997a) and the analysis of mutations generated during DNA replication *in vitro* (reviewed by Kunkel 1992), it is likely that small changes in the number of repeats within simple repetitive tracts are a consequence of DNA polymerase slippage. According to this model (Figure 1), during replication of a repetitive tract, a transient dissociation of the primer and template DNA strands allows a misaligned reassociation of the two strands (Streisinger *et al.* 1966). This misalignment results in a loop of one or more unpaired repeat units. If this DNA mismatch remains uncorrected, the repetitive tract will contain more repeats (if the DNA loop is on

the primer strand) or fewer repeats (if the loop is on the template strand) following a second round of replication. One prediction of this model is that two different classes of mutants leading to destabilized microsatellites should be observed: those deficient in the repair of DNA loops and those affecting DNA polymerase or cofactors resulting in increased rates of DNA polymerase slippage.

Mutations in genes required for DNA mismatch repair elevate greatly microsatellite instability in *E. coli* (Levinson and Gutman 1987; Strauss *et al.* 1997), yeast (Strand *et al.* 1993, 1995; Sia *et al.* 1997b), and mammalian cells (reviewed by Kolodner 1996; Modrich and Lahue 1996). These results suggest that small loops forming as a result of DNA polymerase slippage are efficiently removed by the DNA-mismatch-repair system, and this interpretation is supported by studies of the properties of DNA mismatch repair *in vitro* (Kolodner 1996; Modrich and Lahue 1996). We previously demonstrated that yeast strains with a mismatch-repair deficiency as a consequence of *msh2* or *mlh1* null mutations had elevated levels of repeat instability for repetitive tracts with repeat unit lengths between 1 and 13 bp (Sia *et al.* 1997b). No effect of these mutations was observed for repetitive tracts in which the repeat unit length was 16 bp or greater. We defined those tracts with repetitive units of 13 bp or less as microsatellites and those with tracts with repeat units >15 bp as minisatellites.

Although loss of mismatch-repair function does not affect minisatellite instability, mutations in two yeast genes whose products are components of the DNA replication system destabilize both minisatellites and micro-

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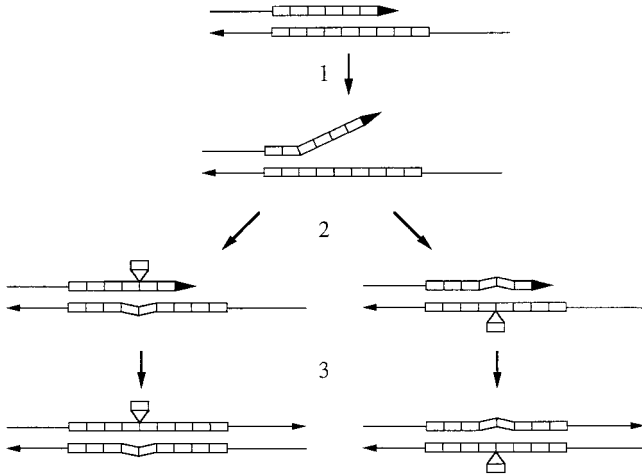E-mail: tompetes@email.unc.edu

Figure 1.—Alterations in the length of simple repetitive DNA sequences by DNA polymerase slippage. Complementary DNA strands are shown with repeat units shown by rectangles. Top and bottom strands represent primer and template strands, respectively; a small arrow represents the 3′ end of the template strand and a triangle indicates the 3′ end of the primer strand. In step 1, primer and template strands transiently dissociate. In step 2, misalignment upon reannealing of the strands results in loops on either the template strand (right) or primer strand (left). In step 3, DNA synthesis is completed. If the DNA loops are not repaired, a deletion (right) or addition (left) of repeat units would result following the subsequent round of DNA synthesis.

satellites. A null mutation of the *RAD27* gene, which encodes a nuclease involved in Okazaki fragment maturation, results in an 11-fold enhancement in the rate of minisatellite instability (Kokoska *et al.* 1998) and 20- to 200-fold destabilizing effects on microsatellites containing various repeat unit lengths (Johnson *et al.* 1995; Kokoska *et al.* 1998; Schweitzer and Livingston 1998). In addition, yeast strains bearing a temperature-sensitive allele of the *POL3* gene (*pol3-t*; Tran *et al.* 1995, 1996), an essential gene encoding the catalytic subunit of DNA polymerase δ, exhibit a 13-fold increase in the rate of minisatellite instability and small effects on microsatellite instability (Kokoska *et al.* 1998).

The proliferating cell nuclear antigen (PCNA) has a role in both DNA replication and DNA repair. PCNA is a replication processivity factor that binds DNA polymerase δ and ε to DNA during replication (Krishna *et al.* 1994) and is required for SV40 replication *in vitro* (Prelich *et al.* 1987a,b). In yeast, PCNA is encoded by *POL30* (Bauer and Burgers 1990), and mutant *pol30* alleles that lead to *in vitro* and *in vivo* defects in DNA replication have been characterized (Ayyagari *et al.* 1995; Amin and Holm 1996). In addition to these replication-defective alleles, some *pol30* alleles affecting UV sensitivity (indicating a deficiency in *in vivo* nucleotide excision repair) or the level of spontaneous mutation (indicating an *in vivo* deficiency in DNA mismatch repair) have been identified (Ayyagari *et al.* 1995; Amin

and Holm 1996). Because some of these mutant strains do not exhibit a DNA replication defect (Ayyagari *et al.* 1995), the role of PCNA in DNA repair is separable from its role as a DNA polymerase processivity factor. As described below, these dual roles of PCNA are also demonstrated by analysis of the *pol30-52* allele.

Yeast cells bearing the cold-sensitive *pol30-52* allele arrest as large budded cells at the restrictive temperature, consistent with a defect in DNA replication (Ayyagari *et al.* 1995). This mutation also causes a 5-fold increase in cycloheximide resistance in a forward mutation assay (Ayyagari *et al.* 1995) and an 80- to 150-fold increase in the instability of a poly GT tract (Johnson *et al.* 1996; Umar *et al.* 1996). The level of dinucleotide tract instability in a strain with both the *pol30-52* and a *mlh1* mutation is about the same as that observed in the single-mutant strains (Umar *et al.* 1996). This result suggests that the effect of *pol30-52* on dinucleotide tracts is primarily on DNA mismatch repair. This conclusion is also supported by the physical interactions between PCNA and both Mlh1p and Msh2p, as well as the requirement for PCNA in an *in vitro* mismatch-repair assay using human cell extracts (Umar *et al.* 1996; Gu *et al.* 1998). One interpretation of these data is that PCNA associates with the DNA-mismatch-repair machinery to ensure the preferential removal of mismatched bases from the newly synthesized strand (Umar *et al.* 1996).

To clarify further the role of PCNA in DNA replication and mismatch repair, we examined the effect of the *pol30-52* mutation on the stability of microsatellites in which the size of the repeat unit varies from 1 to 8 bp and a minisatellite containing 20-bp repeat units. We found that the *pol30-52* mutation destabilized both microsatellites and minisatellites, suggesting that the destabilizing effect of this mutation on simple repetitive DNA is not limited to an effect on DNA mismatch repair.

MATERIALS AND METHODS

Strains and plasmids: All yeast strains were derived from AMY125 (α *ade5-1 leu2-3 trp1-289 ura3-52 his7-2*; Strand *et al.* 1993). MS71 is a *LEU2* derivative of AMY125 (Strand *et al.* 1995). MS71-*pol3-t* was described previously (Kokoska *et al.* 1998). LS1-59 (α *pol30-52* derivative of MS71) was constructed by transformation of MS71 with *Eco*RI-treated pBL241-52 (Ayyagari *et al.* 1995; supplied by P. Burgers, Washington University); this plasmid contains a 1.1-kb *Kpn*I-*Spe*I fragment of *pol30-52* inserted into the *Kpn*I-*Spe*I site of the *URA3*-containing integration vector pRS306. Following selection and purification of Ura+ transformants, we performed the second step of the transplacement using medium containing 5-fluoroorotate (5-FOA; Boeke *et al.* 1984). The resulting Ura− isolates were screened for the presence of *pol30-52* by screening for the inability to grow at 14°, because strains with this mutation are cold sensitive (Ayyagari *et al.* 1995).

To obtain isogenic haploid strains with both the *pol30-52* and *pol3-t* mutations, we sporulated and dissected a diploid (RJK127-4) that was heterozygous for these mutations; in addition, this diploid contained a plasmid (pEAS20) used to assay minisatellite stability. The diploid was constructed by mating

LS1-59::pEAS20 and a derivative of MS71-*pol3-t* (RJK109-3) that had been switched to the opposite mating type.

To determine the effect of *pol30-52* on various types of repetitive DNA sequences, we transformed LS1-59 with the *TRP1*-containing assay plasmids pMD28, pSH44, pBK3, pBK10, and pEAS20 (Henderson and Petes 1992; Sia *et al.* 1997b), selecting for Trp⁺ derivatives. Each of these plasmids contains a microsatellite or minisatellite inserted in-frame within the coding sequence of the *URA3* gene. The sequences of the repeats within these plasmids are pMD28, $(G)_{18}$; pSH44, $(GT)_{16}$; pBK3, $(CAACG)_{15}$; pBK10, $(CAATCGGT)_{10}$; pEAS20, $(CAACGCAATGCGTTGGATCT)_3$.

Analysis of simple repeat instability in *pol30-52* strains: The derivatives of LS1-59 that contained the assay plasmids pMD28, pSH44, pBK3, pBK10, or pEAS20 were phenotypically Ura⁺, because the repetitive sequences within the plasmids were inserted in-frame within *URA3.* Alterations in tract length (either additions or deletions) resulting in an out-of-frame insertion can be selected by plating the cells on medium containing 5-FOA (Henderson and Petes 1992; Sia *et al.* 1997b). To determine the rate of instability for each of the repetitive tracts, we measured the frequencies of 5-FOA^R cells in 12–20 independent cultures. These frequency measurements were converted to rates using the method of the median (Lea and Coulson 1949), as described previously (Henderson and Petes 1992). The rate data shown in Table 1 are based on rates calculated for two independently derived transformants; for each pair of transformants, the rate measurements agreed within a factor of two.

Additional details of the rate measurements (for example, the composition of the media) have been previously described (Wierdl *et al.* 1997). For most of the experiments, the *pol30-52* strains were grown at 30° before analysis on 5-FOA-containing medium also at 30°. For comparisons of the rate of instability of the single *pol30-52* and *pol3-t* mutants with the double-mutant *pol30-52 pol3-t* strain, the cells were grown and assayed for 5-FOA resistance at 25°.

The types of alterations generated in independent 5-FOA^R isolates were determined by PCR or DNA sequence analysis, as described previously (Sia *et al.* 1997b).

Analysis of minisatellite instability in *pol30-52 pol3-t* strains: As described above, *pol30-52 pol3-t* double-mutant haploid strains were constructed by sporulating and dissecting the doubly heterozygous diploid strain RJK127-4; the diploid was grown, prior to sporulation, in medium lacking tryptophan to force retention of the pEAS20 plasmid. The resulting spores were grown for 5 days at 25°. About one-quarter of the spore colonies were very small. We measured the number of cells in each colony (with a hemocytometer), as well as the number of viable cells (by plating the cells on rich growth medium). We also examined the ability of these strains to grow at 37° and 14°, the restrictive temperatures of strains with *pol3-t* and *pol30-52* mutations, respectively. We found that the very small spore colonies had the double mutation.

With continued subculturing of the double-mutant strains, we found that faster-growing derivatives appeared. To reduce the probability of such secondary mutations, we modified the procedure for monitoring tract stability to eliminate subculturing. We suspended spore colonies in sterile water, plated one aliquot on solid medium lacking tryptophan (to score the number of viable cells that retained the pEAS20 assay plasmid), and a second aliquot to 5-FOA-containing medium (to monitor alterations in the minisatellite in pEAS20). Colonies on these two plates were scored after 7 days at 25°; the ability of the cells to grow at 14° and 37° was also determined. From the frequency of 5-FOA^R cells in each spore colony, we calculated a rate of instability using the method of the median as described above; the rate calculation for the double-mutant strain was based on nine spore colonies.

Statistical analyses: Confidence limits of 95% on rate estimates were assigned by the method described previously (Wierdl *et al.* 1996). For comparisons of the types of tract alterations in different mutant backgrounds, we used the Fisher exact test [Instat 1.12 (GraphPad Software) for the Macintosh].

RESULTS

Microsatellite instability in the *pol30-52* mutant: Umar *et al.* (1996) previously showed that a yeast strain with the *pol30-52* mutation has a 150-fold elevation in the frequency of alterations in a dinucleotide microsatellite. As described in the Introduction, several lines of evidence suggested that this mutation causes a defect in DNA mismatch repair. To define more completely the role of PCNA in mismatch repair, we examined the effects of *pol30-52* on the stability of microsatellites in which the size of the repeat unit ranged from 1 to 8 bp. We previously showed that each of these microsatellites exhibits enhanced levels of instability in mismatch-repair-deficient *msh2* and *mlh1* null mutant strains (Sia *et al.* 1997b).

To measure microsatellite instability, we used the plasmid-based frameshift assay described previously (Henderson and Petes 1992; Sia *et al.* 1997a). For this assay, a microsatellite sequence is inserted in-frame within the plasmid-borne coding sequence of a fusion protein that has *URA3* activity. Strains transformed with this plasmid are phenotypically Ura⁺ and are sensitive to the drug 5-FOA. Thus, alterations in tract length that result in an out-of-frame insertion can be selected on medium containing 5-FOA. DNA sequence and/or PCR analysis of the plasmid-borne microsatellite tracts in the 5-FOA^R strains confirms that most of these strains have tract alterations of the expected types (Henderson and Petes 1992).

The rates of microsatellite instability in wild-type, *msh2::Tn10LUK*, and *pol30-52* strains are shown in Table 1; *msh2::Tn10LUK* is a null mutant allele of *MSH2*, resulting from an insertion of a transposable element into the coding sequence (Reenan and Kolodner 1992). All classes of repeats were destabilized by the *pol30-52* mutation. The most striking effects were seen with microsatellites bearing 1- or 2-bp repeat units, which were destabilized 1800- and 380-fold, respectively. The rate obtained for the dinucleotide repeats is in agreement with that reported previously by others (Johnson *et al.* 1996; Umar *et al.* 1996). The effects of the mutation on the instability of microsatellites containing 5- and 8-bp repeat units were more modest (20-fold effect for the 5-bp repeats and 67-fold effect for the 8-bp repeats).

The rates of tract instability observed in the *pol30-52* strains, in general, were similar to those previously seen in an *msh2::Tn10LUK* strain (Table 1; *msh2* data from Sia *et al.* 1997b), consistent with the conclusion that the *pol30-52* mutation causes a DNA-mismatch-repair defect. There was an exception to this generalization. The

TABLE 1

Rates of microsatellite and minisatellite instability in wild-type, *msh2::Tn10LUK*, and *pol30-52* strains

Repeat size × repeat no.[a]	Relevant genotype[b]	Rate of 5-FOA^R cells in independ. expts. (10^{-5}) [c]	Average rate of tract changes (10^{-5}) [d]	Fold increase *vs.* wild type
1 × 18	Wild type	0.59 (0.36–1.3)	0.67	1
		0.80 (0.70–1.1)		
		0.61 (0.40–0.68)		
	msh2	3900 (1900–5500)	4100	6100
		4200 (1100–6000)		
	pol30-52	1500 (920–2300)	1200	1800
		910 (670–2000)		
2 × 16.5	Wild type	0.49 (0.37–0.97)	0.48	1
		0.51 (0.36–0.76)		
	msh2	150 (120–170)	160	340
		150 (110–270)		
		180 (170–380)		
	pol30-52	160 (120–260)	180	380
		190 (120–210)		
5 × 15	Wild type	3.4 (2.9–7.7)	3.0	1
		2.8 (2.4–5.5)		
	msh2	57 (39–98)	49	16
		50 (39–110)		
		43 (32–55)		
	pol30-52	59 (45–120)	61	20
		68 (47–110)		
8 × 10	Wild type	0.96 (0.72–1.2)	0.94	1
		1.1 (0.74–2.0)		
	msh2	14 (9.2–22)	11	12
		11 (4.7–22)		
	pol30-52	62 (38–140)	62	65
		62 (40–87)		
20 × 3	Wild type	6.9 (5.4–11)	7.4	1
		7.8 (6.5–10)		
	msh2	5.5 (4.4–11)	6.9	1
		15 (11–19)		
		12 (10–18)		
	pol30-52	57 (45–69)	43	6
		29 (19–53)		

Data on the rates of instability in wild-type and *msh2* strains were from Sia *et al.* (1997b) and Kokoska *et al.* (1998).

[a] The first number is the number of base pairs per repeat unit, and the second is the number of repeats within the plasmid-borne tract.

[b] All strains are isogenic except for the indicated mutation.

[c] Numbers outside of parentheses represent rates calculated by the method of the median based on measuring the frequency of 5-FOA^R cells in 10–20 independant cultures. Numbers in parentheses are 95% confidence limits.

[d] The rates of 5-FOA^R derivatives were multiplied by the proportion of those derivatives in which the repetitive tract had a size alteration (always >0.8) to yield the rates of tract alterations. These rates were averaged for each strain.

pol30-52 mutation destabilizes the octanucleotide repeat to a significantly greater extent than does the null *msh2* mutation. The interpretation of this effect will be discussed in detail below.

Additional support for the role of PCNA in mismatch repair was provided by the observed spectra of alterations within the microsatellites of 5-FOA-resistant isolates from the *pol30-52* strain. The numbers of isolates with each type of alteration (+, addition; −, deletion) observed for each class of repeat were mononucleotide

(1, +1 repeat; 14, −1 repeat; 3, −2 repeats), dinucleotide (11, +1 repeat; 13, −1 repeat; 1, −10 repeats), pentanucleotide (1, +2 repeats; 3, +1 repeat; 1, no change in number of repeats; 10, −1 repeat; 7, −2 repeats; 1, −4 repeats; 1, > −5 repeats), and octanucleotide (1, +1 repeat; 19, −1 repeat; 1, −2 repeats; 1, −4 repeats). As observed for *msh2* strains, most of the alterations represented single-unit additions and deletions. In addition, for most of the microsatellites (with the exception of the dinucleotide), there was a signifi-
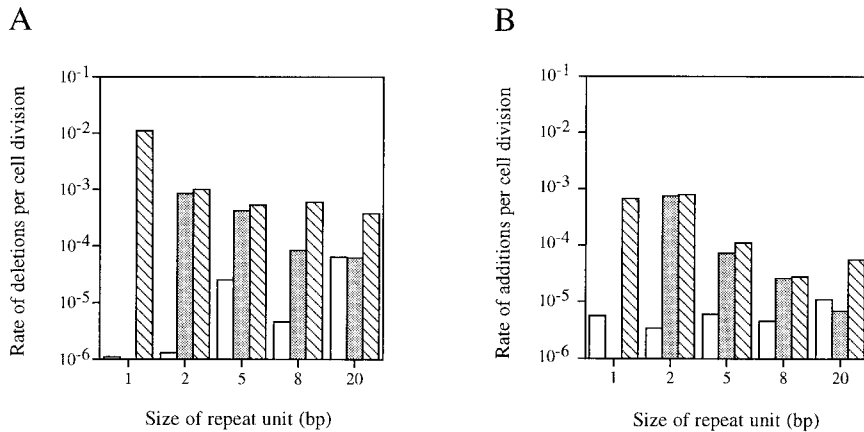
A

B

Figure 2.—Rates of deletions (A) and additions (B) of microsatellites and a minisatellite in wild-type and mutant strains. Rates are given for isogenic wild-type (unshaded bars), *msh2::Tn10LUK* (dotted bars), and *pol30-52* strains (hatched bars). Rates are calculated by multiplying the overall rate of tract alterations by the fraction of alterations observed as deletions or additions. Data from Table 1 were used for calculating the rates for the *pol30-52* strain. Rates for the wild-type and *msh2* strains were based on measurements reported previously (Sia *et al.* 1997b; Kokoska *et al.* 1998). No rate measurement could be calculated for the mononucleotide tract in the *msh2* strain because the types of alterations were not determined.

cant excess of deletions compared to additions in the *pol30-52* strain. These comparisons are summarized in Figure 2.

One significant difference between *msh2::Tn10LUK* and *pol30-52* strains is the types of microsatellite alterations observed with the octanucleotide microsatellite. For the *pol30-52* strain, we found 21 deletions and 1 other type of alteration (an addition); in previous studies of the same microsatellite in an *msh2* strain (Sia *et al.* 1997b), we observed 13 deletions and 8 other types of alteration (4 additions and 4 mutational changes elsewhere in the *URA3* gene). This difference is statistically significant ($P < 0.01$ by Fisher exact test) and confirms the conclusion that the phenotype of *pol30-52* is not identical to that observed for *msh2* for the octomer microsatellite. We conclude that *pol30-52* destabilizes certain microsatellites by at least two mechanisms, one involving DNA mismatch repair and one involving a different mechanism.

The effects of *pol30-52* on microsatellite stability are different qualitatively and quantitatively from those observed in strains with the *rad27* mutation. Although this mutation also destabilizes microsatellite sequences, most of the altered microsatellites in *rad27* strains have additions rather than deletions (Johnson *et al.* 1995; Tishkoff *et al.* 1997; Kokoska *et al.* 1998).

Minisatellite instability in *pol30-52* strain: To provide additional evidence that the effect of the *pol30-52* mutation was not limited to loss of the DNA-mismatch-repair system, we analyzed minisatellite stability in the *pol30-52* strain containing the assay plasmid pEAS20 (three 20-bp repeats inserted with the *URA3* fusion gene). As shown in Table 1, the *pol30-52* mutation destabilized the minisatellite 6-fold. We previously demonstrated that the stability of this repeat is unaffected by mutations in the *MSH2* (as shown in Table 1) or *MLH1* genes (Sia *et al.* 1997b), but is destabilized by the *pol3-t* (11-fold) and *rad27* (13-fold) mutations (Kokoska *et al.* 1998). This result shows that the effects of the *pol30-52* muta-

tion on simple repetitive DNA sequences are not limited to DNA mismatch repair.

We also analyzed the types of minisatellite tract alterations in the *pol30-52* strain. Most (20 of 23) of the tract changes were deletions. Of the deletions, 17 involved one repeat and 3 involved two; we also found 3 single-repeat insertions. These numbers are significantly different from those observed in a *pol3-t* strain ($P < 0.02$ by Fisher exact test) with the same assay plasmid (9 single-repeat deletions and 11 double-repeat deletions; Kokoska *et al.* 1998). In addition, the numbers of tract additions and deletions in the *pol30-52* strain are strikingly different ($P < 0.0001$ by Fisher exact test) from those observed in a *rad27* strain with pEAS20 (27 additions, 13 deletions; Kokoska *et al.* 1998). Thus, although mutations affecting three different components of the DNA replication machinery destabilize minisatellite sequences, the types of alteration observed in these mutants are qualitatively different.

Effects of combined *pol30-52 pol3-t* mutations on growth rate and minisatellite instability: Because *pol30-52* and *pol3-t* have different effects on minisatellite instability, we constructed haploid strains with both mutations to analyze further the relationship between these two mutants. The double-mutant strain was constructed by sporulating a diploid that was doubly heterozygous for the two mutations. We found that the spore colonies containing the double mutation grew extremely slowly relative to the wild-type or single-mutant strains. After 5 days of growth at 25°, the average number of cells in each genotype (as determined with the hemocytomer) were $2.6 \times 10^7 \pm 0.8$ (wild type), $1.6 \times 10^7 \pm 0.4$ (*pol3-t*), $6.5 \times 10^6 \pm 2.2$ (*pol30-52*), and $1.2 \times 10^5 \pm 0.4$ (*pol30-52 pol3-t*); averages were based on at least nine colonies of each genotype and 95% confidence limits are indicated. For the same samples, we also measured the number of viable cells in each colony by plating suspensions of the colonies on rich growth medium, resulting in the following data: $2.0 \times 10^7 \pm 0.2$ (wild type),

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TABLE 2

**Rates of minisatellite instability in wild-type, *pol3-t*, *pol30-52* and *pol30-52 pol3-t*
double-mutant haploid strains after growth at 25°**

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Genotype	Rate of instability/cell division[a]	Normalized rate of instability
Wild type	9.0×10^{-5} ($7.7-12 \times 10^{-5}$)	1
pol3-t	2.2×10^{-4} ($1.6-3.1 \times 10^{-4}$)	3
pol30-52	4.1×10^{-4} ($3.5-6.4 \times 10^{-4}$)	5
pol30-52 pol3-t	2.1×10^{-3} ($1.2-5.3 \times 10^{-3}$)	23

Unlike the data shown in Table 1, these data were obtained in experiments in which the strains were grown at 25°.

[a] Rates for the wild-type, *pol3-t*, and *pol30-52* strains were based on an analysis of 20 independent cultures. Fluctuation analysis for the *pol30-52 pol3-t* strain was performed as described in materials and methods. Numbers in parentheses represent 95% confidence intervals.

$8.7 \times 10^6 \pm 2.8$ (*pol3-t*), $3.2 \times 10^6 \pm 1.4$ (*pol30-52*), and $9.8 \times 10^3 \pm 5.4$ (*pol30-52 pol3-t*).

To reduce the likelihood of acquiring secondary mutations affecting growth rate and minisatellite stability, we examined the frequency of 5-FOA^R cells in individual spore colonies without subcloning of the strains. These frequency measurements were converted to a rate by the method of the median. Since we analyzed the rate of instability of the double mutant in cells grown at 25° (a compromise between the restrictive temperatures of *pol3-t* and *pol30-52*), we also measured the rates of instability of pEAS20 in the wild-type and single-mutant strains grown at 25°. We found that two single mutants elevated instability of the minisatellite about 3- to 5-fold, whereas the double mutant strain had a 23-fold elevation (Table 2).

When the phenotype of the double-mutant strain is compared to that of the two single mutants, the interaction may be epistatic (equivalent to the effect of the more severe mutant phenotype), additive (equal to the sum of the effects of the two single mutants), or synergistic (greater than the sum of the effects of the two single mutants) (reviewed by Haynes and Kunz 1981). The Table 2 data are most consistent with a synergistic or additive interaction in the double-mutant strain.

We also examined the types of minisatellite alterations in 11 independent 5-FOA^R derivatives of the *pol30-52 pol3-t* strain. We found 6 tracts with one-repeat deletions, 3 with two-repeat deletions, 1 with a one-repeat addition, and 1 with no change. The ratio of single-repeat to two-repeat deletions in the double-mutant strain appears intermediate between the ratios found for *pol30-52* strain (17 one-repeat deletions, 3 two-repeat deletions) and *pol3-t* (9 one-repeat deletions, 11 two-repeat deletions; Kokoska *et al.* 1998).

DISCUSSION

Our analysis of the stability of various classes of repetitive DNA sequences in *pol30-52* strains indicates that this PCNA mutation has two different effects. As expected from previous studies (Johnson *et al.* 1996; Umar *et al.* 1996), the mutation destabilized repetitive DNA sequences as a consequence of a DNA-mismatch-repair deficiency. The mutation had an additional destabilizing effect for repetitive sequences with large (≥ 8 bp) repeat units that (as discussed below) is likely to reflect increased DNA polymerase slippage. The rates and types of alterations observed in simple repetitive DNA tracts in *pol30-52* strains were different from those observed in strains with mutations affecting other components of the DNA replication machinery. These conclusions will be discussed in more detail below.

The effect of mutations in yeast DNA-mismatch-repair genes on simple repetitive DNA sequences depends on the length of the repeat unit and the specific repair gene defect (reviewed by Sia *et al.* 1997a). Strains that lack DNA mismatch repair as a consequence of null *msh2*, *mlh1*, and *pms1* mutations have elevated instability for repetitive sequences with repeat unit lengths of ≤ 13 bp. Mutations in these genes, however, do not affect repetitive DNA sequences with repeat units >15 bp. In the context of the DNA polymerase slippage model (Figure 1), these results indicate that DNA loops ≥ 15 bp cannot be repaired by the DNA-mismatch-repair system, although DNA loops ≤ 13 bp are susceptible to repair by this system (Sia *et al.* 1997b).

As shown in Table 1, the destabilizing effects of the *pol30-52* mutation are indistinguishable from those of *msh2* for microsatellites with repeat units of 1, 2, or 5 bp. The rate of alterations for the microsatellite with the 8-bp repeat unit, however, is significantly elevated in *pol30-52* compared to *msh2::Tn10LUK*, and the types of changes are significantly different. In *pol30-52* strains, there are significantly more deletions than in *msh2* strains (Figure 2). In addition, the *pol30-52* mutation, unlike *msh2*, destabilized the 20-bp minisatellite sequence. These results demonstrate that PCNA has two roles in the control of the stability of repetitive DNA sequences, a function in DNA-mismatch repair [as previously demonstrated by Umar *et al.* (1996) and Johnson *et al.* (1996)], and a function that is independent

of the known DNA-mismatch-repair mechanism. The function of PCNA in DNA mismatch repair may be related to directing the mismatch-repair complex to correcting errors on the newly synthesized strand (Umar *et al.* 1996).

We suggest two models to explain the destabilizing effect of *pol30-52* that is independent of its deficiency in DNA mismatch repair. First, PCNA may be required for two different DNA loop repair correction systems. One of these systems is the known DNA-mismatch-repair system (reviewed by Kolodner 1996). The other (as yet uncharacterized) system only recognizes large DNA loops (>5 bp) and competes for the repair of these loops with the known repair system. Thus, the *pol30-52* mutation would be expected to have a stronger effect than *msh2::Tn10LUK* only for repetitive DNA sequences with a repeat unit length >5 bp. Two points are relevant to the suggestion of an undiscovered DNA-loop-repair system. First, Miret *et al.* (1996) reported the existence of a DNA-loop-binding activity in yeast strains deficient in Msh2p, Msh3p, and Msh4p. Second, DNA loops are repaired efficiently in meiosis by a mechanism that is different from the classical DNA repair system and that is, at least partly, Msh2p-independent (Kirkpatrick and Petes 1997).

Second, as expected for a mutation in a protein required for DNA polymerase processivity, the *pol30-52* mutation may increase the rate of DNA polymerase slippage. Previously, we found that the rate of instability in *msh2::Tn10LUK* strains was much higher for mononucleotide microsatellites than for microsatellites with larger repeat units (Sia *et al.* 1997b), suggesting that the primer and template strands might undergo small dissociations much more frequently than large dissociations. We suggest that PCNA has a role in preventing extensive (>8 bp) primer-template dissociations, but not small (<8 bp) dissociations. Consequently, the effects of *pol30-52* (in excess of those expected for the DNA-mismatch-repair deficiency) are observed only for larger repeat units (8 and 20 bp). Alternatively, the *pol30-52* mutation may elevate DNA polymerase slippage for all microsatellites. If only small (≤5 bp), but not large, DNA loops could be removed by the proofreading exonuclease activities associated with DNA polymerase δ or ε, then results consistent with our observations would be observed. At present, there is no evidence that the proofreading exonuclease has this specificity.

Although the current evidence does not allow an informed choice among the models discussed above, we favor the DNA polymerase slippage models, because they are most consistent with the known properties of PCNA. The protein encoded by *pol30-52* has a single amino acid substitution (Ser_{115} to Pro_{115}) in the region controlling subunit interactions (Krishna *et al.* 1994). In solution, formation of the mutant protein into the active trimer is defective and the mutant protein fails to stimulate synthesis by DNA polymerase δ or ε *in vitro*

(Ayyagari *et al.* 1995). Because yeast strains containing the *pol30-52* mutation are viable (unlike strains with the null mutation), the mutant protein must not be completely defective *in vivo.* Thus, the mutant protein may increase the frequency of DNA polymerase dissociation from the template; this dissociation, in turn, could result in an increased frequency of extensive primer-template strand separation events as required by our second model.

Several types of evidence indicate that the effects of *pol30-52* on the stability of simple repetitive DNA sequences are distinct from the effects of mutations in other components of the DNA replication machinery. First, the types of changes observed for the minisatellite assay plasmid were significantly different in comparisons of *pol30-52* with either *pol3-t* (temperature-sensitive mutant of DNA polymerase δ; Tran *et al.* 1995, 1996) or *rad27* (deficient in FEN-1 endonuclease required for Okazaki fragment processing; Murante *et al.* 1996) strains. Most of the altered minisatellite sequences in the *pol30-52* strain were single-unit deletions. The *pol3-t* mutation results in substantially more two-unit deletions (Kokoska *et al.* 1998), whereas *rad27* primarily causes single-unit insertions (Kokoska *et al.* 1998). Second, unlike *pol30-52*, most of the other DNA replication mutants destabilize simple repetitive DNA sequences with short (≤5 bp), as well as long, repeat units by a mechanism that is independent of DNA mismatch repair. These mutants include *pol3-t* (Kokoska *et al.* 1998), *pol3-01* (a mutation in the proofreading exonuclease domain of DNA polymerase δ; Strand *et al.* 1993), *rad27* (Johnson *et al.* 1995; Kokoska *et al.* 1998; Schweitzer and Livingston 1998), and *rfc1* (a mutation in the large subunit of DNA replication factor C; P. Greenwell, R. Kokoska and T. D. Petes, unpublished data; Y. Xie *et al.* 1999). Although these results suggest that mutations in different components of the DNA replication machinery have different effects on the stability of repetitive DNA sequences, we cannot rule out the possibility that some of these effects might reflect allele-specific differences.

Our observation that the types of minisatellite alterations are different in *pol3-t* and *pol30-52*, as discussed above, suggests that their destabilizing effects might reflect different mechanisms. This hypothesis is consistent with the observation that the rate of instability in the double-mutant strain is considerably higher than in either single-mutant strain. In addition, the double-mutant strain has a much slower growth rate than either single-mutant strain. Although this lack of epistasis is usually interpreted as demonstrating that the two mutations affect different pathways, because the mutants were not nulls, this argument is suggestive, but not conclusive.

Regardless of the specific mechanisms involved, modest increases in minisatellite instability may be a general feature of yeast strains that have mutations in genes that

encode components of the DNA replication machinery. Although in human cells the most dramatic alterations within minisatellites occur in meiosis rather than in mitosis (Jeffreys *et al.* 1994), enhanced minisatellite instability has been observed within somatic cells of certain human tumor cell lines (Armour *et al.* 1989; Nagel *et al.* 1995). Our observations suggest the possibility that these human tumor cell lines may have mutations in genes that encode DNA polymerase or accessory factors involved in DNA replication.

In summary, the *pol30-52* mutation destabilizes simple repetitive DNA sequences in a unique way. As reported previously (Umar *et al.* 1996), the mutation results in a DNA-mismatch-repair deficiency. For repetitive DNA sequences with repeat units of ≥8 bp, the mutation has an additional destabilizing effect, possibly related to an increased level of DNA polymerase slippage.

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