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Pathway correcting DNA replication errors in *Saccharomyces cerevisiae*

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Mutation of predicted 3′→5′ exonuclease active site residues of *Saccharomyces cerevisiae POL3* DNA polymerase (δ) or deletion of the *PMS1* mismatch repair gene lead to relative (to wild type) spontaneous mutation rates of ~130 and 41, respectively, measured at a *URA3* reporter gene inserted near to a defined replication origin. The *POL3* exonuclease-deficient mutant *pol3-01* generated most classes of single base mutation in *URA3*, indicating a broad specificity that generally corresponds to that of the *PMS1* system. *pol3-01 pms1* haploid cells ceased growth after a few divisions with no unique terminal cell morphology. A *pol3-01/pol3-01 pms1/pms1* diploid was viable and displayed an estimated *URA3* relative mutation rate of 2×10^4, which we calculate to be catastrophically high in a haploid. The relationship between the relative mutation rates of *pol3-01* and *pms1* was multiplicative, indicating action in series. The *PMS1* transcript showed the same cell cycle periodicity as those of a set of DNA replication genes that includes *POL3*, suggesting *PMS1* is co-regulated with these genes. We propose that the *POL3* 3′→5′ exonuclease and the *PMS1* mismatch repair system act on a common pathway analogous to the *dnaQ→mutHLS* pathway of DNA replication error correction in *Escherichia coli*.

Key words: 3′→5′ exonuclease/mismatch correction/*PMS1*/mutagenesis/yeast

Introduction

An important principle apparent in the correction of DNA replication errors in bacteria is the action of two systems in series, so that their effects are multiplied. This principle seems to apply to the correction of DNA replication errors by the *dnaQ* 3′→5′ editing exonuclease (subunit ϵ of DNA polymerase III holoenzyme) and the *mutHLS* mismatch correction system, systems of broad specificity that together contribute a factor of 10^4-10^5 to replication fidelity (Schaaper, 1988). The prokaryotic 3′→5′ exonuclease is conserved in both structure and activity in the N-proximal regions of yeast *POL3* and *POL2* DNA polymerases and of mammalian DNA polymerase δ, the homolog of yeast *POL3* polymerase (Simon *et al.*, 1991; Morrison *et al.*, 1991; Chung *et al.*, 1991; Zhang *et al.*, 1991). Similarly, a general DNA mismatch correction system appears to be conserved across the phylogenetic spectrum (Holmes *et al.*, 1990; Varlet *et al.*, 1990; Thomas *et al.*, 1991). In yeast, this activity requires *PMS1*, which is structurally related to the prokaryotic *mutL* and *hexB* (B.Kramer *et al.*, 1989; W.Kramer *et al.*, 1989). Consistent with a role in DNA error correction *in vivo*, mutants in *PMS1* or in the 3′→5′ exonuclease active site residues of *POL3* DNA polymerase exhibit a spontaneous mutator phenotype (W.Kramer *et al.*, 1989; Simon *et al.*, 1991).

Here, we test the idea that the action in series of 3′→5′ exonucleolytic editing and mismatch correction is conserved in yeast. We create the *pol3-01* mutant by altering 3′→5′ exonuclease active site residues encoded by *POL3* and make a preliminary examination of its mutational spectrum. We present evidence for a multiplicative relationship between *pol3-01* and *pms1*, indicating action in series and show that *PMS1* appears to be co-regulated with a set of DNA replication genes that includes *POL3*. Finally, we discuss the contribution of this pathway to DNA replication fidelity and the possible additional involvement of the 3′→5′ exonuclease of *POL2* DNA polymerase.

Results

Spontaneous mutation rates of pol3-01 and pms1 mutants

We described previously the creation of a 3′→5′ exonuclease-deficient *POL2* DNA polymerase by altering the Phe-Asp-Ile-Glu-Thr 'ExoI' amino acid sequence motif to Phe-Ala-Ile-Ala-Thr (Morrison *et al.*, 1991). We altered the *POL3* gene to produce the equivalent Phe-Asp-Ile-Glu-Cys to Phe-Ala-Ile-Ala-Cys change and the resulting *pol3-01* mutation was introduced into the yeast chromosome by integration and excision of plasmid YIpAM26 (Figure 1). We constructed a *pms1* mutant by transplacement of the *PMS1* coding region by *LEU2* using plasmid PAM58 (Figure 1). Mutation rates were measured by forward mutation to 5-fluoro-orotic acid resistance (Boeke *et al.*, 1984) of a *URA3* reporter gene inserted near to a defined DNA replication origin, *ARS306* on chromosome III (Figure 1), and/or as reversion of *his7-2*. Mutation rates more representative of the genomic rate are expected from *URA3*, where the target for mutation is the entire gene, than for reversion of *his7-2* where the target may be a single codon. The spontaneous *URA3* mutation rate in the control strain was 1.8×10^{-8} (Table I), similar to the 2.8×10^{-8} reported for *URA3* inserted at the *his3* locus (Lee *et al.*, 1988; Drake, 1991). The relative (to control) *URA3* mutation rates for *pol3-01* and *pms1* were 130 and 41, respectively, (Table I), compared with ~400 and ~30 for mutation to canavanine-resistance reported for similar *pol3* (*cdc2*) and *pms1* mutants, respectively (Simon *et al.*, 1991; Kramer *et al.*, 1989).

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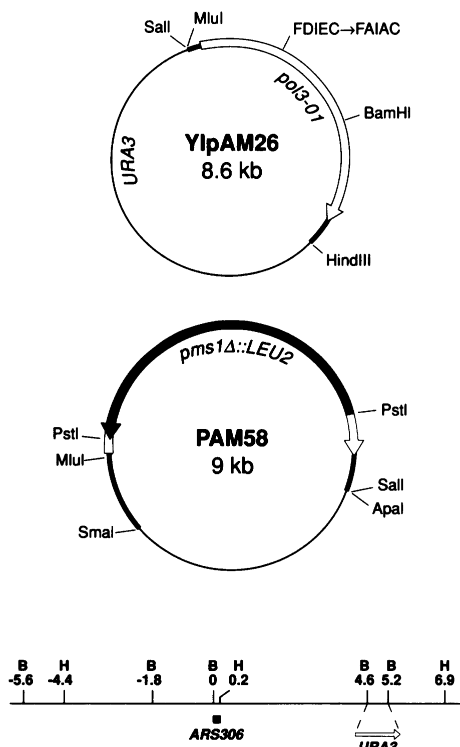


Fig. 1. Structures of plasmids and *ARS306::URA3* construct. Key to plasmids YIpAM26 and PAM58: open arrow, coding region of *POL3* or *PMS1* genes; thick line, flanking yeast DNA; fine line, vector DNA; solid arrow, *LEU2* gene. The location of the DNA encoding the FDIEC→FAIAC change is given (the single letter amino acid code is used). Key to *ARS306::URA3* construct: positions of *BglII* (B) and *HindIII* (H) sites in kb relative to a *BglII* site at the *ARS306* origin (closed box) are given (Deshpande and Newlon, 1993); a 1.1 kb fragment containing the *URA3* gene (open arrow) was inserted as shown. The neighboring *ARS305* and *ARS307* replication origins lie ~38 kb towards the telomere to the right and ~30 kb towards the centromere (*CEN3*) to the left of *ARS306*, respectively (Reynolds *et al.*, 1989; Newlon *et al.*, 1991).

Nucleotide sequence of spontaneous URA3 mutations in a pol3-01 strain

We sequenced the *ura3* gene from 20 independent 5-fluoro-orotic acid-resistant mutants generated in the *pol3-01* haploid. Each mutant had one single base change within the *URA3* coding sequence, with eight of the mutations occurring at three hotspots (Table II). Although the sample size was small, all classes of single base mutation were represented except A·T→T·A and G·C→C·G transversions. The broad specificity of the *POL3* 3′→5′ exonuclease appears to correspond generally to that of the *PMS1* mismatch correction system: as determined from heteroduplex repair experiments, *PMS1* acts with high or intermediate efficiency on single nucleotide loops and single base, except C·C, mismatches (B.Kramer *et al.*, 1989). Interestingly, uncorrected C·C (or G·G) mismatches give rise to G·C→C·G transversions, which were not observed among the sequenced *pol3-01* mutations.

pol3-01/pol3-01 pms1/pms1 diploid

We attempted unsuccessfully to construct a *pol3-01 pms1* haploid and concluded that this double mutant was probably inviable (see below). A double homoallelic *pol3-01/pol3-01 pms1/pms1* diploid, however, was constructed as follows. First, diploid AMY402 was obtained from a cross of AMY401-10C and AMY401-10D, both of which have the genotype *pol3-01 pms1* [*PMS1 URA3*] (i.e. they carry the *PMS1* and *URA3* genes on a plasmid). The median frequency of plasmid loss, measured as resistance to 5-fluoro-orotic acid, was determined for 10 independent cultures of AMY402 grown in YPDA medium. The median frequency was 0.063 for AMY402, compared with 0.36 for a control diploid obtained from a cross of AMY401-10C and AMY360-6A. Analysis of the 5-fluoro-orotic acid-resistant isolates, named AMY405, showed that they had the genotype *pol3-01 pms1* and had therefore lost the *PMS1* plasmid (data not shown). Diploid AMY405 grew slowly, with a doubling time of 5 h, which may explain the relatively low rate of plasmid loss from AMY402. The mutation rate of AMY405 for *his7-2* was $\sim 1.4 \times 10^{-4}$ (Table I). Following integration of *URA3* into the *ARS306* region of one chromosome III copy of AMY405 to create AMY406, the *URA3* mutation rate was measured as $\sim 3.5 \times 10^{-4}$ (Table I). The *ura3* gene from 20 independent 5-fluoro-orotic acid-resistant colonies of AMY406 was amplified by PCR; each generated the expected ~0.9 kb DNA fragment, thus eliminating the possibility that deletion of *URA3* by recombination contributed significantly to the mutation rate (data not shown).

The viability of the *pol3-01/pol3-01 pms1/pms1* diploid shows that there is no intrinsic requirement for the *POL3* 3′→5′ exonuclease and *PMS1* gene product for progression through the cell cycle. However, an estimate of the genomic mutation rate of the *pol3-01 pms1* strain suggests that it is likely to be lethal in a haploid. The *URA3* mutation rate of AMY406 is $\sim 1.9 \times 10^4$-fold higher than that of the *URA3* derivative of CG379 (Table I). (This figure is an approximation from a comparison of strains of different ploidy, although this is likely to have relatively a slight effect; furthermore, isogenicity would appear to be impossible to maintain in a strain with such a high mutation rate.) Extrapolating from *URA3* to the entire genome, the total number of spontaneous mutations per cell division in a *pol3-01 pms1* strain is expected to be $1.9 \times 10^4\ \mu \approx 63$, where μ, the spontaneous mutation rate in a wild type cell, is 0.0033 (Drake, 1991). The number of lethal mutations per haploid cell division is $1.9 \times 10^4\ \mu E/C = 2.4$, where E, the fraction of genes encoding essential functions, is ~0.12 (Goebl and Petes, 1986) and C, the reciprocal of the efficiency of mutant detection, is 3.1 (Drake, 1991). This is likely to underestimate the actual lethality of the mutation rate, since 14% of detectable mutations are expected to cause growth defects (Goebl and Petes, 1986), which may interact synergistically to cause cell death.

Lethality of pol3-01 pms1 in haploids

The inviability of a *pol3-01 pms1* haploid was formally demonstrated by tetrad analysis of spores from a diploid heterozygous for both *pol3-01* and *pms1* mutations (Figure 2). These tetrads each yielded four, three or two viable spores in approximately the ratio 1:4:1, the theoretical ratio for incompatibility of the two mutations, while spore viability in the controls was at least 98% (Figure 2; A.Morrison, unpublished observations). Determination of the spore genotypes showed the expected 2:2 segregation of *pol3-01* and *pms1*, and no viable *pol3-01 pms1* segregant. Viewed by a light microscope after 3 days on YPDA plates, the *pol3-01 pms1* segregants comprised microcolonies of

Table I. Measurement of spontaneous mutation

Genotype[a]	Reversion of *his7-2*		Forward mutation of *URA3*	
	Mutation rate (× 10^8)[b]	Relative rate[c]	Mutation rate (× 10^8)[b]	Relative rate
+ +	0.55 ± 0.23 (15)	1	1.8 ± 0.67 (4)	1
+ *pms1*	85 ± 27 (12)	150	74 ± 36 (3)	41
pol3-01 +	130 ± 30 (9)	240	240 ± 97 (3)	130
+/+ +/+	0.37 ± 0.16 (6)[d]	1	–	–
pol3-01/+ +/+	2.2 ± 1.0 (6)[d]	6	–	–
+/+ *pms1/pms1*	93 ± 13 (6)[d]	250	–	–
pol3-01/pol3-01 +/+	180 ± 120 (4)[e]	490	–	–
pol3–01/+ *pms1/pms1*	890 ± 170 (6)[d]	2400	–	–
pol3-01/pol3-01 pms1/pms1	14 000 ± 5700 (3)[f]	38 000	35 000 ± 3200 (2)[g]	19 000[h]

Spontaneous mutation measured as forward mutation of the *URA3* gene, inserted near the *ARS306* replication origin and/or as reversion of *his7-2* to His^+.

[a]Haploids were CG379 and its *pol3-01* or *pms1* derivatives obtained by gene transplacement.

[b]Results given as mean ± standard deviation (number of determinations) or mean ± range when only two determinations were made. Except where noted, three different isolates of each strain were used.

[c]Relative to the control strain of the same ploidy.

[d]For each genotype, two determinations for each of three isolates were averaged. Diploids were formed by crossing strain CG379 or its *pol3-01* and/or *pms1* derivatives with three different *PMS1* or *pms1* segregants of AMY355. The strains used were as follows. +/+ +/+: CG379×AMY355-6C, -9B or -11D. *pol3-01/POL3* +/+: CG379 *pol3-01*×AMY355-6C, -9B or -11D. +/+ *pms1/pms1*, CG379 *pms1*×AMY355-3D, -7C or -10C. *pol3-01/POL3 pms1/pms1*: AMY270×AMY355-3D, -7C or -10C; before measurement of *his7-2* reversion, these diploids were cultured in YPDA and plated on 5-fluoro-orotic acid to select for loss of the *PMS1 URA3* plasmid contributed by AMY270.

[e]Two determinations for each of two isolates were averaged. Diploids were formed from the crosses AMY410-5B×AMY410-24D and AMY410-22D×AMY410-25B.

[f]Diploid AMY405, obtained by crossing AMY401-10D and AMY401-10C and selecting for loss of the *PMS1 URA3* plasmid by plating cultures grown in YPDA on 5-fluoro-orotic acid.

[g]Diploid AMY406, derived from AMY405 by integration of *URA3* into the *ARS306* region of one chromosome III copy. Two different isolates were used.

[h]Relative to the haploid control strain.

~90 cells on average (16 determinations). Cell types counted from portions of 14 of these microcolonies consisted on average of ~45% single, unbudded cells, 9% budded cells, 27% dumbbells, 12% aberrants and 7% others (usually triple or quadruple cells that had not separated). These morphologies do not suggest failure to complete S phase, since dumbbell-shaped cells would then have been expected to predominate. However, the lack of a unique terminal morphology and high proportion of aberrants is consistent with death from a catastrophically high mutation rate. The 6–7 divisions on average before termination is an upper limit, since products of the *POL3* and *PMS1* genes present in the diploid might persist through several divisions of the germinating spores.

Multiplicative relationship between pol3-01 and pms1

The action of the *POL3* 3'→5' exonuclease and *PMS1* gene product in series on a common pathway, as illustrated in Figure 3ii, is expected to yield a multiplicative relationship between the relative spontaneous mutation rates of their respective mutants. This is described by equation 1 (Materials and methods), in which M_d and M_p here represent the observed relative spontaneous mutation rates of *pol3-01* and *pms1*, respectively, and *e* is the fraction of spontaneous mutations in the control strain that represents replication errors still remaining after action by the *POL3* 3'→5' exonuclease, *PMS1* pathway. Since *e* is unknown, we calculate the minimum relative *URA3* mutation rate of a *pol3-01 pms1* haploid strain as $M_{dp} \geq 130 \times 41 \approx 5 \times 10^3$. The 1.9×10^4 estimated for the double homoallelic diploid (Table I) is good evidence for multiplicity, with $e \approx 0.3$. For *his7-2*, the predicted relative mutation rate is $\geq 490 \times 250 \approx 1.2 \times 10^5$, somewhat greater than but in reasonable agreement with the observed 3.8×10^4, using the values for diploids given in Table I.

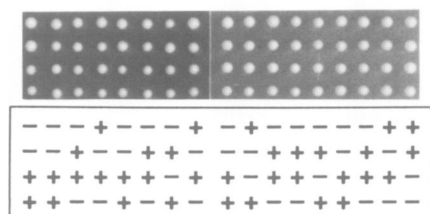
Table II. Spontaneous mutations in the *URA3* gene in the *pol3-01* derivative of CG379

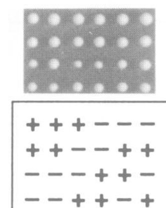
Class	Change	Occurrences	Mutation
Transitions	G·C→A·T	4	G768→A, G926→A(2), G990→A
	A·T→G·C	5	T321→C, T323→C(4)
Transversions	G·C→C·G	0	
	G·C→T·A	1	G566→T
	A·T→T·A	0	
	A·T→C·G	3	T394→G(2), T506→G
Deletions	−1	5	C319, A336, T908, A982, T1003
Insertions	+1	2	427+T, 946+G
Others		0	
Total		20	

Parentheses indicate multiple occurrences.

Multiplicity is easily distinguished from the additivity expected if the two functions act in parallel, non-competing pathways as shown in Figure 3iii: then equation 2 (see Materials and methods) gives a value for $M_{dp} = 130 + 41 - 1 = 170$ using the values for *URA3* mutation in Table I (and similarly for *his7-2* reversion). Furthermore, the *POL3* 3'→5' exonuclease is not required for *PMS1*-dependent mismatch correction (Figure 3i), since the double mutant would then have a relative mutation rate no greater than that of either single mutant.

It remains possible that the two activities act in competing





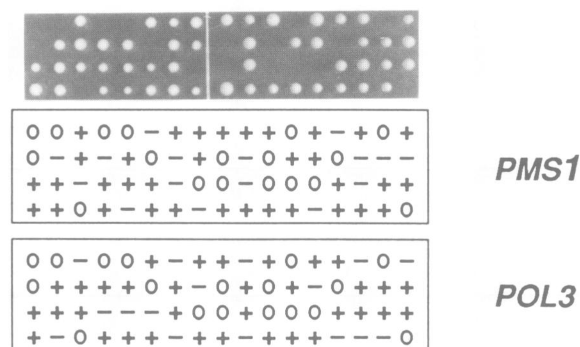


Fig 2. Tetrad analysis of diploid heterozygous for *pol3-01* and *pms1*. Shown are photographs of germinated spores from microdissected tetrads. Genotypes are diagrammed below: +, wild type; −, mutant (*pol3-01* or *pms1*); o, no visible colony. **A.** CG378 *pms1*×CG379; genotypes refer to *PMS1*, determined by replica plating onto plates lacking leucine. **B.** AMY360-8D×CG379; genotypes refer to *POL3*. **C.** AMY360-8D×CG379 *pms1*; genotypes refer to *PMS1* and *POL3*, as labeled.

pathways, as in Figure 3iv, such that one may compensate for the absence of the other and conceivably $M_{dp} >> M_d + M_p$. We have tested this by determining the relationship between the *pol3-01* and *pms1* relative mutation rates in the presence of *POL3*$^+$, which is possible because *pol3* exonuclease-deficient mutants are partially dominant (Simon *et al.*, 1991; Table I). Table I shows the *his7-2* reversion rates of diploids of genotype *pol3-01*/*POL3*, either in a *PMS1*/*PMS1* or *pms1*/*pms1* background. Using these figures, the predicted value for multiplicity is $M_{dp} \geq 6\times250 = 1.5\times10^3$ (here M_d represents the relative mutation rate of *pol3-01*/*POL3*), compared with the observed value of M_{dp} of 2.4×10^3. We then infer that the relationship between *pol3-01* and *pms1* is indeed multiplicative.

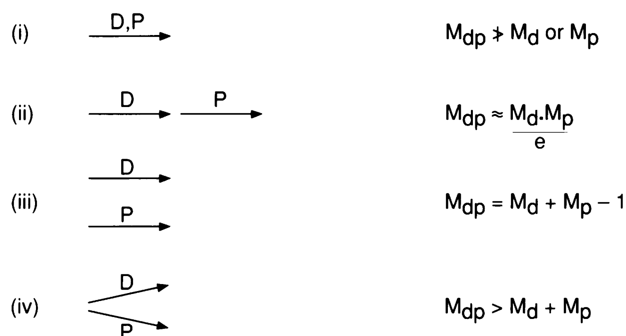


Fig. 3. Four possible relationships between two DNA replication error correction systems. Replication errors (not shown) are corrected by two systems, D and P, with arrows representing steps in a pathway. To the right, the relative (to wild type) spontaneous mutation rate of a strain defective in both D and P (M_{dp}) is expressed as a function of M_d and M_p, the relative spontaneous mutation rates of strains defective in either D or P, respectively. In panel i, both D and P are required for the same step in a pathway and the double mutant is no more defective than a single mutant. Action in series (panel ii), gives rise to a multiplicative relationship between M_d and M_p; in the expression for M_{dp} described by equation (1) in Materials and methods, *e* represents residual replication errors remaining after action of D and P. Action in parallel, non-competing pathways (panel iii) results in additivity of M_d and M_p, given by equation (2) in Materials and methods. Competition between D and P for the same replication errors (panel iv) gives rise to a synergistic relationship between M_d and M_p if the system is not saturated, since P (or D) can compensate for the absence of D (or P).

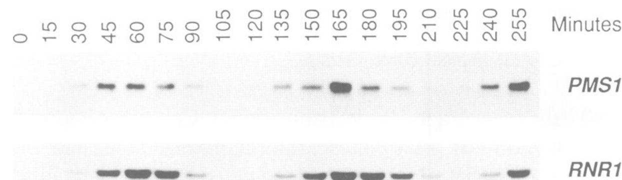


Fig. 4. Steady-state levels of the *PMS1* transcript during the cell cycle. *S.cerevisiae* cells were synchronized by elutriation and aliquots withdrawn at the indicated times. Northern blots were hybridized with a ^{32}P-labeled 2.7 kb *Mlu*I−*Sst*I *PMS1* DNA fragment or a DNA fragment of *RNR1*, which encodes ribonucleotide reductase.

Cell cycle regulation of PMS1 transcript

Inspection of the 5′ upstream region of the *PMS1* nucleotide sequence (W.Kramer *et al.*, 1989) shows that it contains the *Mlu*I cleavage sequence 5′-ACGCGT and the related sequence 5′-ACGCGA beginning 33 and 48 nucleotides, respectively, upstream of the translational start codon. The 5′-ACGCGT sequence is required for the co-ordinate expression of a group of DNA replication genes, including *POL1*, *POL2*, *POL3* and *RNR1* etc, coincident with the onset of S phase (Gordon *et al.*, 1991; Lowndes *et al.*, 1991; McIntosh *et al.*, 1991; Johnston and Lowndes, 1992). Figure 4 shows that the *PMS1* transcript isolated from synchronously growing cells accumulated periodically during the cell cycle in essentially the same way as the transcript of *RNR1* (encoding ribonucleotide reductase), which peaks at the G_1/S boundary. The synchronized cells in the experiment shown in Figure 4 were prepared by elutriation, but we observed the same periodicity using cells synchronized by a feed−starve protocol or using α-factor (data not shown). *PMS1* is thus apparently co-regulated with DNA replication genes, suggesting that it acts during DNA replication.

Table III. Yeast haploid strains

Strain	Genotype
CG379	*MATα leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2*
CG378	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 can1*
AMY270	*MATα leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pol3-01 pms1 [PMS1 URA3]*
AMY360-6A	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 can1*
AMY360-8D	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pol3-01*
AMY355-6C	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2*
AMY355-9B	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2*
AMY355-11D	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2*
AMY355-3D	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pms1 can1*
AMY355-7C	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pms1 can1*
AMY355-10C	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pms1*
AMY401-10C	*MATα leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pol3-01 pms1 can1 [PMS1 URA3]*
AMY401-10D	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pol3-01 pms1 [PMS1 URA3]*
AMY410-5B	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pol3-01*
AMY410-24D	*MATα leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pol3-01*
AMY410-22D	*MATa leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pol3-01*
AMY410-25B	*MATα leu2-3,-112 trp1-289 ura3-52 ade5-1 his7-2 pol3-01*

Discussion

We base the proposal that the *POL3* 3'→5' exonuclease acts on a common pathway with the *PMS1* mismatch correction system on the criteria of multiplicity of the relative mutation rates of their respective mutants, apparently similarly broad specificities for single base mutations *in vivo* and a common cell cycle regulation. Matching, or at least overlapping specificities of two systems are required if they are to act in series, because if their specificities are different, they can act only in parallel and their contributions will be additive, not multiplicative. Co-regulation with DNA replication genes reinforces evidence of a role for *PMS1* during mitotic DNA replication, in addition to its repair of mismatches arising during meiotic recombination (Williamson *et al.*, 1985; Bishop *et al.*, 1987; Lichten *et al.*, 1990). The potential mutations corrected by this pathway are, as with the *Escherichia coli dnaQ→mutHLS* pathway, highly likely to be errors of DNA replication. The contribution to reduction of spontaneous mutations is M_{dp}/e, where M_{dp} is the relative mutation rate of *pol3-01 pms1*, estimated at $\sim 1.9 \times 10^4$. e, the proportion of spontaneous mutations that represents replication errors, is likely to be <1 and may be as low as 0.1 (Quah *et al.*, 1980), in which case M_{dp}/e would equal $\sim 2 \times 10^5$. Using our estimate from *URA3* mutation of $e \approx 0.3$, the contribution of the pathway would be $\sim 6 \times 10^4$.

POL2 DNA polymerase, the ϵ polymerase of *Saccharomyces cerevisiae*, is the other genomic DNA replicase possessing a 3'→5' editing exonuclease that may contribute to DNA replication fidelity (Araki *et al.*, 1991; Morrison *et al.*, 1991). The 3'→5' exonuclease-deficient *pol2-4* mutant has a relative spontaneous *URA3* mutation rate of only ~12, but preliminary evidence suggests that the *POL2* and *POL3* 3'→5' exonucleases act synergistically (Morrison and Sugino, 1992a). The total contribution of 3'→5' exonucleolytic editing might therefore be significantly greater than that estimated for *pol3-01* alone and the total contribtion of the 3'→5' exonuclease→mismatch repair pathway might be similarly greater. If so, a *pol2-4 pol3-01 pms1* triple mutant might be unstable, if not lethal, in a diploid and actual measurement of its mutation rate might require a strain of a higher ploidy.

Our experiments suggest several comments regarding the *E.coli dnaQ→mutHLS* pathway: It is perhaps surprising that the reported $\approx 10^5$ relative mutation rate of a *mutD5* (*dnaQ*) mutant (in rich medium) is not lethal to the cell (Schaaper, 1988). The inference from spontaneous mutational spectra that the *dnaQ* 3'→5' exonuclease primarily removes potential transition mutations and *mutHLS* system removes potential transversions appears puzzling (Wu *et al.*, 1990): to the extent that these specificities do not overlap, the two systems must act not in series but in parallel, even if their actions are consecutive in the temporal sense. Lastly, the 3'→5' exonucleases of *E.coli* DNA polymerases I and II might conceivably compensate for the absence of the *dnaQ* activity, so that a triple 3'→5' exonuclease mutant might have a phenotype even more extreme than that of *dnaQ*.

Materials and methods

Yeast strains

Yeast haploid strains are listed in Table III. CG379 and CG378 were obtained from Craig Giroux. AMY360 segregants were from a cross of CG379 *pol3-01* and CG378, AMY355 segregants from a cross of CG379 and CG378 *pms1*, AMY410 segregants from a cross of CG379 *pol2-4* and AMY360-8D, and AMY401 segregants from a cross of AMY270 and AMY355-7C.

Plasmids

pBL304 (11.05 kb), containing the 3.7 kb *Mlu*I–*Hin*dIII interval of *POL3* DNA cloned as a *Sal*I–*Hin*dIII fragment into the *Sal*I–*Hin*dIII sites of YCp50, was the gift of Peter Burgers. pBLAM1 was derived from pBL304 by replacement of *POL3* by *pol3-01*. YIpAM26 (Figure 1) was generated by cloning *pol3-01* as a 3.7 kb *Sal*I–*Hin*dIII fragment from pBLAM1 into the *Sal*I–*Hin*dIII interval of YIp5. A *PMS1 URA3* plasmid containing the 4 kb *Bgl*II–*Sal*I fragment of *PMS1* inserted in the *Bam*HI–*Sal*I sites of WBK3, which contains *CEN4* (W.Kramer *et al.*, 1989), was obtained from Michael Resnick. *PMS1* was subcloned as a *Sma*I–*Sal*I fragment (*Sma*I immediately precedes *Bam*HI in the polylinker) into the *Sma*I–*Sal*I sites of vector BluescriptM13+ (Stratagene) to generate PAM57. To create PAM58 (Figure 1), the *Pst*I–*Pst*I interval of the *PMS1* coding region in PAM57 was replaced by a 4.1 kb *LEU2 Pst*I fragment from YEp13. A 28 kb plasmid (C1G) containing the *ARS306* DNA replication origin was kindly provided by Thomas Petes. From this a 6.7 kb *Hin*dIII fragment containing *ARS306* was cloned into pBR322 to generate PAM81 (11.1 kb).

PAM92 was derived from PAM81 by replacement of the +4.6 → +5.2 *Bgl*II interval of *ARS306* DNA (Figure 1) by a 1.1 kb *URA3 Bam*HI fragment from plasmid GB310, provided by Craig Giroux.

Construction of the pol3-01 allele

The 0.377 kb *Bsp*EI interval of *POL3* in pBL304 [nucleotides 782–1159 from the *Mlu*I site (Morrison and Sugino, 1992b)], was replaced by a *pol3-01 Bsp*EI fragment generated by PCR and containing the code for the amino acid sequence FAIAC instead of FDIEC (amino acids 320–324, using the single-letter code). PCR reactions used 14 cycles of 94°C, 60°C and 72°C, pBL304 template DNA and oligonucleotides 5′-GTTCCGGAAAGACGC-CAATCCTACCAGCACACGCGATAGCAAAG [complement of DNA matching nucleotides 1357–1314, including the second *Bsp*EI site and in which the wild type GAT and GAG codons for Asp and Glu are changed to GCT and GCG (changed bases underlined), respectively, to encode Ala], and 5′-AAACAGTCTATCTGGGATTATTCCGGAGATACCAAATTA-CCA (corresponding to nucleotides 952–993 and including the first *Bsp*EI site). The *Bsp*EI–*Bsp*EI interval of the resulting pBLAM1 was sequenced on one strand to confirm the sequence expected for *pol3-01*.

Construction of yeast strains

The *pol3-01* allele was introduced into the yeast chromosome by targeted integration (Rothstein, 1983) using YIpAM26 cut with *Bam*HI, followed by selection for excision of the integrated plasmid by growth on 5-fluoro-orotic acid. *POL3* and *pol3-01* isolates were initially distinguished by measuring reversion of *his7-2*. The *POL3*$^+$ isolates had the same *his7-2* spontaneous reversion rate as the original CG379 strain. Strains were also checked by Southern blotting and a PCR test to determine the *POL3*$^+$ or *pol3-01* genotype. *URA3* was inserted near to the *ARS306* replication origin by transforming yeast with a *Pvu*II digest of PAM92 DNA (*Pvu*II sites lie approximately at positions +1.3 and +5.8 using the co-ordinates in Figure 1). The *pms1* gene was inserted by transforming yeast to Leu$^+$ with PAM58 DNA cut with *Mlu*I and *Apa*I (in the polylinker). To obtain AMY270, CG379 *pol3-01* was transformed with the *PMS1 URA3* plasmid and then with the PAM58 DNA digest; Southern blotting showed that *pms1* had replaced the chromosomal *PMS1* gene (replacement of the plasmid *PMS1* gene was not observed). To obtain diploids, haploids were mated and zygotes separated by micromanipulation; diploidy was confirmed by the ability to sporulate. AMY402 was obtained from a cross of AMY401-10D and AMY401-10C. Loss of the *PMS1 URA3* plasmid from AMY402, following growth in YPDA medium and selection for 5-fluoro-orotic acid resistance, yielded AMY405. Integration of *URA3* into the *ARS306* region of one chromosome III copy of AMY405 gave AMY406.

Spontaneous mutation

Spontaneous reversion rates of *his7-2* were measured using the Leningrad test (von Borstel, 1978). Yeast cells grown in YPDA medium to stationary phase were washed, suspended in water at densities of $\sim 10^6-10^7$ cells/ml and applied with a multipronged replicator onto plates containing synthetic medium either lacking or containing 0.8–2 μg/ml histidine. Plates were incubated for 5–10 days at 30°C. The average numbers of mutational events was calculated by the P_0 method or, where few compartment had zero revertants, from the fraction of compartments with *n* revertants, as described by Khromov–Borisow (von Borstel, 1978). For the *pol3-01/pol3-01 pms1/pms1* diploid AMY405, *his7-2* reversion was measured by a fluctuation test as described for *URA3*, except cells were plated on synthetic medium lacking histidine. For *URA3* forward mutation, we used a fluctuation test in which ~100 cells per ml of a stationary phase culture were inoculated into each of 12 YPDA cultures and grown to stationary phase. Cells were counted, washed and plated on 5-fluoro-orotic acid plates and incubated for 5 days. The average numbers of mutational events was calculated either by the P_0 method or from the median number of colonies (von Borstel, 1978).

Nucleotide sequence of ura3 mutations

A 0.925 DNA fragment including the *URA3* gene was amplified from yeast chromosomal DNA by PCR, using 30 cycles of 94°C, 60°C and 72°C and primers representing nucleotides 165–189 and the complement of nucleotides 1089–1065, Genbank database accession number K02206 (Rose *et al.*, 1984). DNA fragments were purified by 1% agarose gel electrophoresis and sequenced using an Applied Biosystems automated sequencer. One strand was sequenced using four primers corresponding to nucleotides 180–199, 385–405, 585–605 and 785–805. Regions containing the mutation and any discrepancies were then sequenced on the opposite strand using primers complementary to nucleotides 1075–1055, 875–855, 675–655 and 405–385. The control *URA3* sequence was identical to that reported (Rose *et al.*, 1984).

Determination of pol3-01 genotype

Yeast DNA was prepared as described by Davis *et al.* (1980), except that no diethylpyrocarbonate was used and DNA was extracted with phenol–chloroform before ethanol precipitation. A 0.4 kb segment of *POL3* DNA amplified by 30 cycles of 94°C, 60°C and 72°C using oligonucleotide primers corresponding to nucleotides 961–985 and the complement of nucleotides 1365–1341, respectively (Morrison and Sugino, 1992b) was challenged with either *Eco*RV or *Bst*UI. The *POL3* nucleotide sequence encoding the 'ExoI' amino acid sequence Asp-Ile-Glu, GAT-ATC-GAG, contains an *Eco*RV site (underlined) but no *Bst*UI site, whereas in the corresponding *pol3-01* sequence, GCT-ATC-GCG, the *Eco*RV site is absent and a *Bst*UI site (underlined) is present.

Calculations

D and P are two error correction systems of similar specificities acting in series (Figure 3ii) and D corrects a fraction $1/f_d$ of errors per cycle, P a fraction $1/f_p$, so that *e* errors remain, while *k* represents other mutations arising independently of the *DP* pathway. M_d, M_p and M_{dp} are the observed relative spontaneous mutation rates in mutants *d*, *p* and *dp*. The residual errors after action of D and P are $e + k = 1$. $M_d = ef_d + k$ and similarly $M_p = ef_p + k$, and $M_{dp} = ef_d f_p + k$, so that

$$M_{dp} = \frac{(M_d - k)(M_p - k)}{e} + k$$

$$\approx \frac{M_d \times M_p}{e} \qquad (1)$$

if both M_d and M_p are much greater than *k*. $M_{dp} = M_d \times M_p$ when $k = 0$ and $e = 1$. If D and P function in parallel, non-competing pathways (Figure 3iii) and E_d and E_p represent errors correctable by D and P, respectively, then $E_d + E_p + k = 1$, $M_d = f_d E_d + E_p + k$ and similarly for M_p and M_{dp}, so that

$$M_{dp} = M_d + M_p - 1 \qquad (2)$$

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