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ORIGINAL PAPER

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Stationary-phase mutations in proofreading exonuclease-deficient strains of the yeast *Saccharomyces cerevisiae*

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Abstract In order to understand the role of yeast polymerases in spontaneous mutagenesis in non-growing cells we have studied the effects of mutations that impair the 3′ → 5′ exonuclease function of polymerases delta (*pol3-01*) and epsilon (*pol2-4*) on the spontaneous reversion frequency of the frameshift mutation *his7-2* in cells starved for histidine. We showed that for each exonuclease-deficient mutant the rate of reversion per viable cell per day observed in stationary-phase cells remained constant up to the 9th day of starvation (while the number of viable cells dropped), and was very similar to that observed in the same mutants during the growth phase. These data suggest that both DNA polymerases are involved in the control of mutability in non-growing cells.

Key words *Saccharomyces cerevisiae* · Stationary-phase mutations · *pol2-4* · *pol3-01* · Exonucleolytic proofreading

Introduction

During the last few years the debate on adaptive mutations in micro-organisms has revived interest in mutations that arise during starvation. Adaptive mutations (directed mutations) were originally defined as mutations that arise only in the presence of selection for such mutations in non-dividing or slowly growing stationary-phase cells. However, in *Escherichia coli* it was found that non-selected mutations may also arise at an unexpectedly high rate during the stationary phase. Several mechanisms have been discovered which may be contribute to stationary-phase mutagenesis; however, there is as yet no unifying model for the process (Rosenberg 1994; Rosenberg et al. 1998; Foster 1999; Lombardo et al. 1999).

In *Saccharomyces cerevisiae*, Hall (1992) and Steele and Jinks-Robertson (1992) reported the occurrence of mutations during starvation and concluded that these are true adaptive mutations. In contrast, a paper by Marini et al. (1999) showed that starvation increases the mutation frequency for both selected and non-selected markers. Actually, little is known about the genetic control of this phenomenon; as far as we know, only one paper has reported an influence of the *cdc2* (*pol3*) gene on mutagenesis in non-growing yeast cells (Baranowska et al. 1995). DNA polymerases delta (polymerase III) and epsilon (polymerase II), encoded by the *cdc2* (*pol3*) and *pol2* genes, respectively, are the only nuclear DNA polymerases in *S. cerevisiae* that possess a 3′→5′ exonuclease activity. Their role in correcting errors in DNA replication in *S. cerevisiae* has been investigated in detail by Morrison and Sugino (1994) and Morrison et al. (1993) in strains in which the exonuclease I domain of the two polymerases was mutated (*pol3-01* and *pol2-4*, respectively; Morrison et al. 1991, 1993), and that are therefore deficient in the 3′→5′ exonuclease activities of these enzymes. Their results showed that impairment of the 3′→5′ exonuclease activity of polymerase delta or epsilon leads to an increase in mutation rates at the loci tested in growing cells, but in their experiments the frequency of mutations arising during the stationary phase was not studied. To obtain further insight into the role of replicative polymerases in stationary-phase mutagenesis, we have evaluated the frequency of reversion at the *his7-2* locus in growing as well as in non-growing cultures of the *pol3-01* and *pol2-4* mutants, using the test developed in yeast by Hall (1992), which allows one to determine the frequency of mutation in the two different phases independently.

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Our results show that the elimination of the 3′→5′ exonuclease activity in DNA polymerases delta and epsilon results in a mutator phenotype not only in growing cells but also in non-dividing cells. This suggests a possible role for polymerase-associated exonuclease activity in error avoidance both during stationary phase and when DNA is being actively replicated.

Materials and methods

Strains

The strains used were CG379-3-29 (LR) [*MATα ade5-1 leu2-3,112 Δura3 bik1::ura3-29 (RL) his7-2 trp1-289 CAN1 lys2-B13*]; *ep2-4* CG379-3-29 (LR) [same as CG379-3-29(LR) but *pol2-4*]; and *de3-01* CG379-3-29 (LR) [same as CG379-3-29(LR) but *pol3-01*] (Shcherbakova and Pavlov 1996; Y. I. Pavlov, P. V. Shcherbakova, K. Bebenek, and T. A. Kunkel, manuscript in preparation).

Estimation of spontaneous mutation rates and surviving fractions

To estimate the frequency of reversion at the *his7-2* allele, which is a –1 frameshift mutation, in *pol3-01* and *pol2-4* strains (Shcherbakova and Kunkel 1999), we used the method described by Hall (1992). A small inoculum of each strain was grown overnight at 30°C in synthetic minimal (SD) medium containing required nutrients, and then aliquots of 100 cells each were plated on SD plates containing a limiting amount of histidine (0.22 mg/ml; SD lim.his.). A total of 50–60 plates were set up for each experiment. After 72 h of incubation at 30°C, small colonies had grown up (about 1 mm in diameter); on these colonies His^+ revertant papillae were detectable from the 5th day on. The numbers of colonies with papillae were counted up to the Day 9, at which point the experiment was terminated to avoid cellular growth due to the release of histidine into the medium by growing papillae and/or by dead cells (Marini et al. 1999). We counted the numbers of colonies with papillae instead of the actual number of papillae because we could not rule out the possibility that more than one papilla might have arisen from the same mutational event in any one colony; this could have caused an underestimation of the mutation frequency. However, even in the case of the *pol3-01* strain, very few colonies were found to form more than one papilla.

The mutation rate during the growing phase was estimated by dividing the number of colonies with papillae on the Day 5 by the number of cells counted on, or the number of cell divisions that had occurred up to, Day 3; hence, papillae were considered to have arisen from a mutational event that had occurred 2 days before (Hall 1992; Marini et al. 1999). The number of divisions that had occurred from the time of plating up to Day 3 was evaluated by counting (in a haemocytometer) the number of cells in a suspension obtained by washing two SD lim.his. plates on Day 3. The plating efficiency was determined by plating about 100 cells on SD + his at the normal concentration (20 mg/ml, SD + his) and it was shown to be about 60–70% for all strains, i.e., the same as that routinely observed for cells growing in liquid SD + his; therefore all cells counted on Day 3 were considered to be viable. We also evaluated the frequency of reversion at the *his7-2* allele by a fluctuation test using the P_0 method (Luria and Delbruck 1943; von Borstel 1978), considering each culture as an independent case.

The mutation rate during stationary phase was estimated by dividing the number of colonies with papillae that arose per day from Day 7 to Day 9 by the total number of viable cells counted 2 days before (see above).

The number of non-growing, viable cells was determined as follows: two plates that did not bear any colonies with papillae (Marini et al. 1999) were washed with 24 ml of saline solution, cells were counted in a haemocytometer and appropriate dilutions were plated on SD + his. The surviving fraction of non-growing cells was calculated as a percentage of the total number of cells capable of growing on SD + his.

To obtain a better estimation of the spontaneous rate of reversion in the wild-type strain, in which the reversion rate of the *his7-2* allele is very low (Morrison and Sugino 1994), we also used the method described by Steele and Jinks-Robertson (1992): 10^8 cells were plated on SD-his plates and His^+ revertants were counted each day through Day 6. To determine the total number of viable cells each day, cells were collected from plates that bore no revertants, counted in the haemocytometer and plated on SD + his, and on SD-his in order to be sure that there were no revertants in the suspension. The reversion rates are given as the number of newly arisen revertant colonies per viable cell per day. With this method also, the revertant colonies were considered to have arisen from a mutational event that had occurred 2 days before.

Results

Spontaneous mutation rates in growing cells

In Table 1 the reversion rates at the *his7-2* locus determined during the growing phase by the method of Hall (1992) are reported for the wild-type strain CG379-3-29 (LR) and for the *pol3-01* and *pol2-4* derivatives of this strain, which are deficient in the 3′→5′ exonuclease activities of polymerases delta and epsilon, respectively. The mutation rates reported were expressed as the number of revertants (colonies with papillae detectable on Day 5) divided by the number of viable cells on Day 3 (see Materials and methods) and were almost identical to the frequencies calculated by the P(0) method (data not shown). It is worthy of note that the rates of reversion at the *his7-2* locus evaluated by Morrison and Sugino (1994) using the Leningrad test (von Borstel 1978) differed markedly between the *pol3-01* and *pol2-4* strains, with the former showing mutation frequencies one order of magnitude higher than the latter; the difference noted in our experiments was not as drastic – less than three fold. This might reflect either the difference in the methods used or clonal variation of mutation rates in the *pol3-01* strain.

Table 1 Reversion rates at the *his7-2* locus in growing wild-type, *pol3-01* and *pol2-4* cells

Strain	Mean number of colonies with papillae observed on Day 5	Reversion rate[a]
Wild type	2	$0.44 \pm 0.07 \times 10^{-8}$
pol3-01	191	$19.40 \pm 1.2 \times 10^{-8}$
pol2-4	52	$7.5 \pm 0.6 \times 10^{-8}$

[a] The data are the means of three experiments and were determined by the method of Hall (1992). The total number of viable cells was 4.7×10^8 for the wild-type strain, 9.03×10^8 for *pol3-01* and 6.9×10^8 for *pol2–4*. The rate of reversion is expressed as the number of colonies with papillae observed on Day 5/the total number of divisions that had occurred up to Day 3. The standard errors of the mean are indicated

The frequency of reversion in wild-type, growing cells, determined by the method of Steele and Jinks-Robertson (1992), was 0.35×10^{-8} (mean of two experiments).

Spontaneous reversion rates in non-growing cells

In non-growing wild-type cells the spontaneous reversion rates were estimated by the Hall (1992) method and by the technique described by Steele and Jinks-Robertson (1992). Here we report only the data obtained with the latter method (Table 2) because it allowed us to evaluate small increases in *his7-2* reversion rate in non-growing wild-type cells. When the uncertainties arising from the small numbers of revertants obtained are taken into account, we observe a small increment in the reversion rates on Days 4, 5 and 6, as expected on the basis of the work of Hall (1992), Steele and Jink-Robertson (1992) and Marini et al. (1999). In all cases, we considered that the revertant colonies arose from mutational events that had occurred 2 days before, that is in cells starved for histidine for 2, 3 and 4 days, respectively (see the data for surviving fractions in Table 2). Indeed the first detectable colonies were observed 2 days after plating, notwithstanding the overcrowding of cells on the plates.

Table 2 Reversion rates and surviving fractions for non-growing wild-type cells

Surviving fraction (%)[a]	Reversion rate[b]
19.0 (2 days)	0.35×10^{-8} (Day 4)
6.5 (3 days)	0.88×10^{-8} (Day 5)
3.0 (4 days)	1.50×10^{-8} (Day 6)

[a] The values given are the means of two experiments (about 40 plates were checked in each experiment). The time after plating is indicated in parentheses (see Materials and methods)
[b] The reversion rates are given as the number of newly arisen revertant colonies/viable cell/day, and were determined by the method of Steele and Jinks-Robertson (1992).The rate of appearance of revertant clones during the log phase in the two experiments was 0.35×10^{-8}

The spontaneous mutation rates in non-growing *pol3-01* and *pol2-4* exonuclease-deficient strains are shown in Table 3; they were determined by the method of Hall (1992). It can be seen that new colonies with papillae arose with a constant (daily) frequency up to Day 9 in both strains and that the rate per day was very similar to the one obtained in growing cells, which, it should be recalled, is given in terms of the numbers of colonies with papillae per cell division (see Table 1). The similarity between the reversion rates in growing and non-growing cells raises the possibility that the revertants we observed in non-growing cells are actually slowly growing mutants that had arisen during the log phase of growth. However, we do not believe that this is the case because these mutants grew as well as the wild type when tested on SD-his plates; moreover, the *his 7-2* allele reverts only due to intragenic frameshift mutations (Shcherbakova and Pavlov 1996), and the resulting revertants are not slow growing. The reversion rate on Day 4 is not reported because the cells counted in the haemocytometer were still budding and their number increased between the 3rd and the 4th day; i.e., these cells were still in the growing phase; after this time, the surviving fraction decreased at the same rate in the wild type and the mutant strains (Table 4). Colonies observed under the microscope up to the 9th day did not show any evidence of multiplication due to cannibalism or to excretion of histidine from papillae (Marini et al. 1999); therefore we assumed that cells did not resume growth up to Day 9.

Discussion

Much attention has recently been devoted to the understanding of spontaneous mutagenesis in non-dividing cells. The term ''stationary-phase mutations'' for such mutations is preferable to ''adaptive mutations'' because in bacteria it has been definitively shown that stationary-phase mutations are not directed, in a Lamarckian sense, to the gene under selection, but, rather, occur in multiple unselected genes. The genetic control of stationary-phase mutations has been extensively studied in *E. coli*,

Table 3 Rates of reversion of the *his7-2* allele in non-growing *pol3-01* and *pol2-4* cells

Strain	Day after plating	Mean number of colonies with papillae[a]	Reversion rate[b]
pol3-01	Day 7	42	$18\times10^{-8}\pm6$
	Day 8	24	$14\times10^{-8}\pm5.7$
	Day 9	11	$16\times10^{-8}\pm2$
pol2-4	Day 7	13	$6.0\times10^{-8}\pm0.3$
	Day 8	7.5	$6.9\times10^{-8}\pm0.6$
	Day 9	4.0	$7.0\times10^{-8}\pm0.7$

[a]The data are the means of three experiments
[b]The reversion rates are reported as the number of newly arisen colonies with papillae/number of viable cells counted 2 days before (see Materials and methods). The standard errors of the mean are reported. For the *pol3-01* strain the total number of viable cells was 2.54×10^8, 2×10^8 and 0.55×10^8 on Days 7, 8, and 9, respectively; for the *pol2-4* the corresponding values were 2.07×10^8, 1.14×10^8 and 0.67×10^8

Table 4 Surviving fraction in non-growing cells (Hall's method)

Days after plating	Surviving fraction (%)[a]		
	Wild type	*pol3-01*	*pol2-4*
Day 3	100	100	100
Day 5	18 ± 3.2	16.1 ± 2.8	18 ± 1.7
Day 6	11 ± 1.1	12 ± 1.3	10 ± 1.8
Day 7t	9 ± 0.6	4.2 ± 0.9	6 ± 0.4

[a] The values are the means of three experiments. The standard errors of the means are reported; all cells were considered to be viable on Day 3 (100%, see Materials and methods). Data for Day 4 are not reported, because cells were found to be in transition from the growing to the non-growing phase at this point (see Results), and these data were not considered in the estimation of reversion rates in non-growing cells

and many genes which are involved in stationary-phase mutagenesis have been described (for reviews see Foster 1999; Lombardo et al. 1999). In the context of our study it is relevant to mention that in *E. coli* it has been shown that the Pol II gene (*polB*$^+$) is involved in stationary-phase mutagenesis; indeed, cells with a *polB* deletion were shown to have a mutator phenotype for stationary-phase mutations of a frameshift allele carried on an episome, implying a role for Pol II in non-dividing cells (Escarcellar et al. 1994). Moreover, Foster et al. (1995) showed that an exonuclease-deficient mutant, *polBex1*, increases the incidence of stationary-phase mutations on an episome in non-dividing cells under lactose selection. In the yeast *S. cerevisiae* the role of numerous DNA polymerases (Friedberg and Gerlach 1999; Hübscher et al. 2000) in stationary-phase mutagenesis was not previously studied.

In yeast, only two polymerases, delta and epsilon, are known to have an intrinsic 3′→5′ exonuclease activity (Morrison et al. 1991; Simon et al. 1991; Morrison and Sugino 1994), which could operate to correct errors during the stationary phase. The possibility exists that they could correct errors introduced by other polymerases which do not have any proofreading activity of their own; indeed, it has been shown that in some wild-type strains there is an, albeit small, increase in mutation rates in non-growing cells (Steele and Jinks-Robertson 1992; Marini et al. 1999; this paper), and this could be due to the action of an error-prone DNA polymerase.

Among the growing family of yeast DNA polymerases, a good candidate to explain the higher mutation rates in non-growing wild-type cells is the DNA polymerase eta, coded by the *rad30* gene. This polymerase, like α, has recently been shown to be relatively error-free in bypassing thymine dimers but replicates undamaged DNA rather inaccurately (Johnson et al. 1999; Matsuda et al. 2000).

To obtain insight into the involvement of replicative DNA polymerases in non-growing yeast cells, we have studied whether – as has been shown for *E. coli* (Foster et al. 1995) – the polymerase-associated exonuclease activity is involved in stationary-phase mutagenesis. We have used the mutant *pol3-01* and *pol2-4* strains, obtained by mutating both Asp and Glu residues in the exonuclease I domain of polymerases delta and epsilon to Ala residues (Morrison et al. 1991; 1993). These authors showed that the *pol3-01* and *pol2-4* mutations increase the spontaneous rate of mutation by two and one order of magnitude, respectively, measured as forward mutation to *ura3* and reversion of the frameshift mutation *his7-2*. There data were obtained using the Leningrad test (von Borstel 1978); with this method mutants were scored up to Day 10 on selective plates, where mutants could have arisen in growing as well as non-growing cells. Therefore in their study it was not possible to compare the mutation rates in the two phases. To achieve this, we have used the method described by Hall (1992).

Our results show that both *pol3-01* and *pol2-4* retain their mutator phenotype also in non-growing cells, suggesting that the delta and epsilon polymerases are involved in correcting errors in stationary-phase cells of yeast.

It is worth noting that the frequency of reversion of the *his7-2* allele in non-growing cells was constant with time throughout the experiment, although we do not at present have any explanation for this observation. It could be argued that DNA synthesis occurs at a constant rate in non-growing yeast cells. In this regard, it should be pointed out that no data on DNA synthesis in yeast non-growing cells are available; however, the data obtained for *E. coli* suggest that no more than 20% of its genome is turned over in 24 h under stationary-phase conditions, and it has been estimated that this value would account for less than 10% of stationary-phase mutations (Tang et al. 1979; Foster 1993; Foster 1999; Bridges 1996); this could suggest that the rate of substitutions per nucleotide should be very high in non-growing cells.

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