JOURNAL OF BACTERIOLOGY, Nov. 1991, p. 6807–6810
0021-9193/91/216807-04$02.00/0

Vol. 173, No. 21

The Spectrum of Spontaneous Mutations in a *Saccharomyces cerevisiae* Uracil-DNA-Glycosylase Mutant Limits the Function of this Enzyme to Cytosine Deamination Repair

KIMBERLY J. IMPELLIZZERI, BLAKE ANDERSON, AND PETER M. J. BURGERS*

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

Received 3 June 1991/Accepted 3 September 1991

Uracil-DNA-glycosylase has been proposed to function as the first enzyme in strand-directed mismatch repair in eukaryotic organisms, through removal of uracil from dUMP residues periodically inserted into the DNA during DNA replication (Aprelikova, O. N., V. M. Golubovskaya, T. A. Kusmin, and N. V. Tomilin, Mutat. Res. 213:135–140, 1989). This hypothesis was investigated with *Saccharomyces cerevisiae*. Mutation frequencies and spectra were determined for an *ung1* deletion strain in the target *SUP4*-o tRNA gene by using a forward selection scheme. Mutation frequencies in the *SUP4*-o gene increased about 20-fold relative to an isogenic wild-type *S. cerevisiae* strain, and the mutator effect was completely suppressed in the *ung1* deletion strain carrying the wild-type *UNG1* gene on a multicopy plasmid. Sixty-nine independently derived mutations in the *SUP4*-o gene were sequenced. All but five of these were due to GC→AT transitions. From this analysis, we conclude that the mutator phenotype of the *ung1* deletion strain is the result of a failure to repair spontaneous cytosine deamination events occurring frequently in *S. cerevisiae* and that the *UNG1* gene is not required for strand-specific mismatch repair in *S. cerevisiae*.

Strand-directed mismatch repair depends on a mechanism which allows the organism to distinguish between the parental and the newly replicated DNA strand during DNA replication. This form of repair is best understood for *Escherichia coli* (11, 23). In methyl-directed DNA mismatch repair, the strand to be repaired is identified by its temporary lack of methylation at GATC sites during DNA replication. The strand specificity of this MutH,L,S-dependent repair system is provided by the MutH protein, which introduces a nick on the unmethylated strand of a hemimethylated GATC site (11). Evidence that the nick itself provides the signal for repair on that strand follows from in vitro studies and from *E. coli* transfection experiments with persistently nicked DNA containing mismatches (20, 21).

Strand-specific mismatch repair systems have also been identified in vivo in *Streptococcus pneumoniae* and in extracts from human or *Drosophila* cells, and again there is evidence indicating that the presence of a nick on one of the strands suffices to guide the repair system to that strand (5, 13). Finally, mismatch repair dependent on the products of the *PMS* genes has been shown to exist in the yeast *Saccharomyces cerevisiae*, although strand specificity for this repair has not yet been established (16, 24). Inasmuch there is significant homology between the *E. coli mutL*, the *S. pneumoniae hexB*, and the *S. cerevisiae PMS1* mismatch repair genes, it is likely that the repair pathways have been conserved in these organisms (17).

Except in the case of methyl-directed mismatch repair in *E. coli*, it is still unclear by which mechanism the breaks necessary for strand-specific repair of the newly replicated strand are generated in these other organisms. Because of the nature of DNA replication, the lagging strand could in principle provide the proper breaks for strand targeting, but this would not be so for the leading strand (12). For this reason, it has been proposed that nicks in the newly replicated DNA could be generated by the incorporation of dUMP from dUTP into the DNA followed by the sequential action of uracil-DNA-glycosylase and an apyrimidinic endonuclease to form a small gap (1, 29).

Uracil is introduced into DNA by two distinct mechanisms (for a review, see reference 29). DNA polymerases can incorporate dUMP instead of dTMP into DNA during DNA replication, resulting in a dU:dA base pair. In addition, cytosine residues present in the genome can undergo spontaneous or induced deamination to uracil, producing a dG:dU base pair, which is a mutagenic event. In either case, repair is initiated by excision of uracil by the uracil-DNA-glycosylase. Uracil-directed mismatch repair in eukaryotes appears as a feasible model for the following reasons. First, incorporation of dUMP into the DNA is nonmutagenic and occurs, at least in mammalian cells, at levels high enough to provide periodic nicks for strand targeting (10). Deoxyuridine levels in DNA isolated from a yeast strain deficient for uracil-DNA-glycosylase were only about 0.1% or less (3), but such low levels are still consistent with a role for uracil excision in targeting the mismatch repair system to the newly replicated strand. Second, uracil-DNA-glycosylase levels are increased in mammalian cells induced to undergo proliferation (30). Similarly, expression of the *UNG1* gene in *S. cerevisiae* is regulated during the cell cycle in exactly the same fashion as are yeast replication genes, with steady-state mRNA levels rising manyfold in late G_1 and early S and decreasing again late in the S phase of the cell cycle (13a). In agreement with this observation is the presence of an *MluI* cell cycle element in the upstream regulatory region of this gene (25).

Previously, we have isolated a yeast strain deficient for uracil-DNA-glycosylase (3). This mutant, carrying the

* Corresponding author.

ungl-1 allele, showed a 5- to 100-fold increase of spontaneous mutations in selected markers (3). Because of the proposed participation of uracil excision in strand-directed mismatch repair and the unusually high spontaneous mutation rates in the *ungl-1* strain, we decided to determine the rates and types of mutations for a yeast strain in which the *UNG1* gene had been deleted. As a target for the isolation of spontaneous mutation events we used the *SUP4*-o tRNA gene of *S. cerevisiae*. An efficient system has previously been developed to select forward mutations in this gene (26). Using this assay system, we have found no evidence implicating uracil-DNA-glycosylase in strand-specific mismatch repair.

TABLE 1. Spontaneous mutations in the *SUP4*-o gene

Expt no.	pPY91	Mutation frequency (10^{-6})[a] in:	
		MKP-op	PY32-p
1	−	1.2	22
2	−	1.4	28
	+	1.6	1.9

[a] Median spontaneous mutation frequencies were determined from 50 independent cultures each in experiment 1 and from 20 cultures each in experiment 2. Red canavanine-resistant colonies were counted, and the numbers were multiplied with the fraction of those colonies that were also Lys$^-$ (see Materials and Methods for details). The frequencies were corrected for residual growth of cells after plating (see Materials and Methods).

MATERIALS AND METHODS

Strains and plasmids. The haploid, repair-proficient strain MKP-o (*MATα can1-100 ade2-1 lys2-1 ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901*) was obtained from C. N. Giroux (National Institute of Environmental Health Sciences) (26). This strain was transformed with the integrating plasmid pBA102, which was constructed as follows. A 5.1-kb yeast genomic *Bam*HI fragment containing the *UNG1* gene was cloned into the *Bam*HI site of plasmid yIp5, which contains the *URA3* gene for selection in *S. cerevisiae* (28); this was followed by deletion of a 0.91-kb *Pvu*I-*Sac*II fragment internal to the insert (25). This deletion removed 85% of the coding region and all of the gene which showed homology with other uracil-DNA-glycosylases (25). Transformants were selected by plating on uracil omission media, and isolated colonies were purified by replating. Integration at the chromosomal *UNG1* locus was verified by Southern analysis of the transformants. The integrated plasmid was then evicted by plating on 5-fluoroorotic acid medium (2) followed by Southern analysis and uracil-DNA-glycosylase assays to identify the deletion mutant PY32 (like strain MKP-o but *ungl-Δ900*). Extracts from PY32 were completely deficient for uracil-DNA-glycosylase activity (25). Strains MKP-o and PY32 were transformed with plasmid YCpMP2, giving MKP-op and PY32-p, respectively. YCpMP2 is a centromer-containing shuttle plasmid carrying the *URA3* gene for selection in *S. cerevisiae* and the *SUP4*-o gene, an ochre suppressor allele of a yeast tyrosine tRNA gene (26). Under selective pressure, i.e., when growing in uracil omission media, plasmid retention in both strains was 90%. In addition, these YCpMP2-containing strains were transformed with pPY91, a yEP-based plasmid carrying the *TRP1* gene as a selectable marker and the *UNG1* gene at a plasmid copy level of about 30 (25). Under selective growth conditions, over 99% of the cells retained this plasmid.

Isolation and analysis of *SUP4*-o mutants. This was essentially carried out as described previously (26). Briefly, strain MKP-o and its *Δung* derivative PY32 carry three ochre-suppressible mutations in chromosomal loci that confer canavanine resistance (*can1-100*), are red because of adenine auxotrophy (*ade2-1*), and are auxotrophic for lysine (*lys2-1*). All of these phenotypes are suppressed by the *SUP4*-o gene present on plasmid yCpMP2, i.e., plasmid-containing strains are white, prototrophic for adenine and lysine, and sensitive to canavanine. Selection for canavanine-resistant colonies which are red, i.e., Ade$^-$, followed by screening for those colonies that are also Lys$^-$ almost exclusively produces mutants with changes in the *SUP4*-o gene. Cells (MKP-op or PY32-p) were grown to saturation on minimal medium with amino acids and adenine but lacking uracil, diluted to about 100 ml in 50 separate cultures, and again grown to saturation. Cells were then diluted and plated on minimal medium supplemented with adenine, all amino acids except arginine, and canavanine. Canavanine was supplemented at 80 μg/ml rather than 30 μg/ml (26) to limit outgrowth of cells after plating. Red colonies (representing about 5% of the total number of canavanine-resistant colonies for the wild type and about 2% for the mutant strain) appearing after 4 days of growth at 30°C (followed by 1 week of storage at 4°C to enhance the appearance of the red pigment) were counted, and a representative fraction was replated on appropriate medium to score for lysine auxotrophy. About 80% of the red canavanine-resistant colonies were also Lys$^-$ and were considered to be mutants in the *SUP4*-o gene. Total DNA was isolated from independently derived mutations in strain PY32-p (i.e., one isolate per separate culture), and the plasmid was recovered in *E. coli* DH5 (*supE44 hsdR17 recA1 gyrA96 thi-1 relA1*). Double-stranded YCpMP2 plasmid was isolated from this strain, and the *SUP4*-o gene was sequenced by the dideoxy-chain termination method.

RESULTS AND DISCUSSION

Selection of *SUP4*-o mutants. The *SUP4*-o tyrosine-inserting ochre-suppressing tRNA gene is an excellent target for mutational analysis (see Materials and Methods and reference 26). Forward mutations are easily detected and, because the gene (including a 14-nucleotide intron) is only 89 nucleotides long, mutations are easily determined by sequencing. Mutations in at least 69 of 75 exon sites in the *SUP4*-o gene have been isolated by this selection procedure, and for most sites all three possible substitution mutations have been identified (9, 15, 19). This argues against a bias in deriving a mutational spectrum when this gene is used as a target.

The median frequency of spontaneous mutations at the *SUP4*-o locus was 1.2×10^{-6} for the wild-type strain and 22×10^{-6} for the isogenic *ungl* deletion mutant, an 18-fold increase in mutation frequency (Table 1). The corresponding mutation rates were 3.8×10^{-7} and 4.1×10^{-6} mutation events per generation, respectively (6). The mutator phenotype of the *Δungl* mutant was completely abolished when the mutant strain was transformed with a multicopy plasmid carrying the *UNG1* gene, whereas the spontaneous mutation frequency of the wild-type strain remained unaffected by this transformation (Table 1). These experiments confirm our earlier results that the presence of the *ungl* mutation confers a fairly strong mutator phenotype upon *S. cerevisiae* (3).

Analysis of *SUP4*-o mutations. A collection of 69 independently derived *SUP4*-o mutants isolated in the *ungl* deletion strain PY32-p were sequenced. The sequence changes are given in Fig. 1, and these are compared with a subset (i.e., all

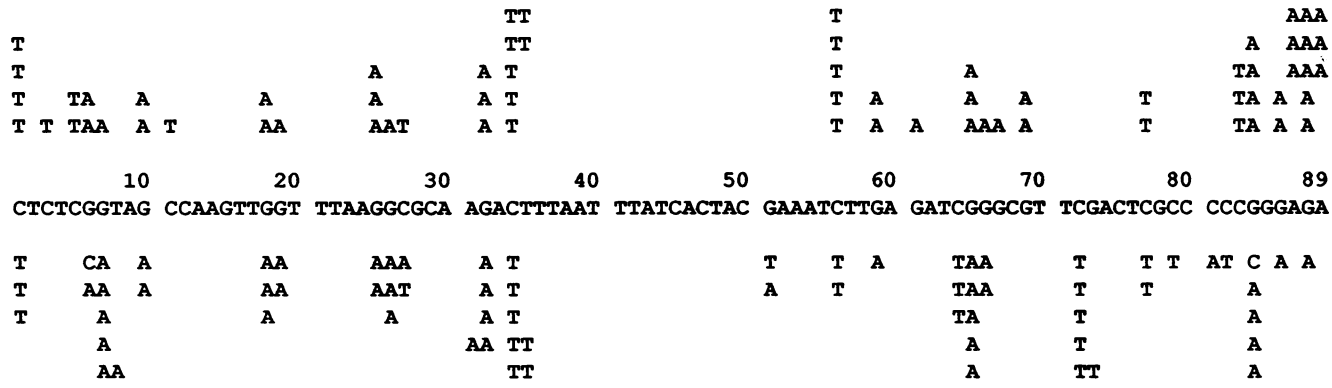

FIG. 1. Distribution of spontaneous base pair substitutions in the *SUP4*-o gene. The coding strand is shown in the 5′→3′ direction. The 14-bp intron extends from nucleotide 40 through nucleotide 53 (14). GC→AT transitions isolated in the wild-type strain MKP-op are from Kunz et al. (19) and are shown above the tRNA gene. All of the mutations isolated in PY32-p (Δ*ungl*) are shown below the gene. One tandem double substitution (GC→AA) occurred at nucleotides 26 and 27.

GC→AT transitions) of spontaneous mutations previously collected in wild-type strain MKP-op by Kunz et al. (19). Because PY32-p and MKP-op are completely isogenic, the mutation spectrum obtained for the mutant can be compared directly with the considerable data base available for MKP-op (19).

Except for one tandem double-base-pair substitution, all spontaneous mutations which arose in the *ungl* deletion strain were single-base-pair substitutions. GC→AT transitions accounted for 65 (93%) of the 70 substitutions. In comparison, in the wild-type strain, GC→AT transitions accounted for 20.1% of the total number of spontaneous mutants isolated (19). If we assume that the calculated 11-fold increase in mutation rate in the Δ*ung* strain is entirely due to a 50-fold increase in GC→AT transitions, the percentage of these transitions should increase from 20.1 to 93% of the total mutations. This is exactly what was observed. Thus, within the quantitative limits of the experiment, mutation events other than GC→AT were not increased in frequency.

The increase in spontaneous mutations in methyl-directed mismatch repair mutants in *E. coli* is predominantly the result of an increase in transitions, with AT→GC occurring twice as frequently as GC→AT (27). Because the mismatch substrate specificity for *PMS*-dependent mismatch repair in *S. cerevisiae* resembles that of the *E. coli* system (16), it is likely that it functions in the avoidance of similar types of mutations. Excluding the unlikely possibility that premutational replication errors by the polymerase are predominantly GC→AT (e.g., see reference 18), the specific large increase in GC→AT transitions in the Δ*ungl* strain is inconsistent with a loss of strand-directed mismatch repair in the mutant strain but is completely consistent with a repair defect of cytosine deamination lesions.

Of the 65 GC→AT transitions, 26 were C→T changes on the nontranscribed strand and 39 were C→T changes on the transcribed strand. This is comparable to the strand distribution of C→T mutations in the wild-type strain and agrees well with the number of genetically detectable C→T mutations on each strand (Fig. 1, Table 2). In contrast, in an *E. coli ung-1* strain the majority of C→T changes were observed on the nontranscribed strand (7). Since deamination of cytosines occurs predominantly when the DNA is in single-stranded form (19, 22), Fix and Glickman concluded, from results obtained with *E. coli*, that the nontranscribed strand would have more of a single-stranded character during transcription (7). The lack of strand bias in *S. cerevisiae* may reflect the fact that both strands display a similar single-stranded character during transcription. Alternatively, deamination may predominate during other processes, e.g., replication, which involve single-stranded DNA intermediates.

Finally, no specific context effect of neighboring sequences was detected in the mutations isolated in the *ungl* deletion strain. GC→AT transitions occurred as frequently in regions rich in G:C base pairs as they did in regions rich in A:T base pairs (Fig. 1). For *E. coli*, it had been observed that cytosine deamination was favored on the 3′ side of three or more A:T base pairs (7).

Cytosine deamination in *S. cerevisiae*. Spontaneous deamination of cytosines, which causes mutations in strains defective for uracil-DNA-glycosylase, is a much more frequent event in *S. cerevisiae* than in *E. coli*. The increase in frequency of GC→AT transitions measured in the *lacI* gene in an *E. coli ung-1* strain is 8×10^{-7}, corresponding to an increased mutation rate per generation of 1.8×10^{-7} in this gene (6, 7). Since the *lacI* gene has 68 sites in which a GC→AT transition produces a selectable phenotype (7), the average increased mutation rate per cytosine residue per cell division in the *ung-1* strain is 2.7×10^{-9}. In contrast, in *S. cerevisiae* the increase in mutation rate in the *ungl* deletion strain is 3.7×10^{-6}, for a total of 33 scorable sites in the *SUP4*-o gene, corresponding to an average mutation rate of 1.1×10^{-7} per cytosine residue per cell division. It is not clear to what the 40-fold increase in the rate of deaminations per cell division can be ascribed. The slower rate of transcription in *S. cerevisiae* in comparison to that in *E. coli* may

TABLE 2. Strand distribution of GC→AT transitions

Strand	Detectable C→T positions[a]	No. of C→T transitions in[b]:	
		MKP-op	PY32-p
Nontranscribed	13	25 (8)	26 (9)
Transcribed	20	42 (17)	39 (15)

[a] Number of positions at which a C→T transition has been genetically detected (15, 19).

[b] Numbers in parentheses are the numbers of positions at which C→T transitions were found.

keep the DNA in single-stranded form for extended periods, increasing the chance for cytosine deamination (8, 22), but the chemical environment in the nucleus of the yeast cell may also be more conducive to cytosine deamination than that in *E. coli*.

In conclusion, uracil excision of dUMP residues incorporated by the DNA polymerase during DNA replication has been proposed to generate the nick on the newly replicated strand which signals strand-specific mismatch repair in eukaryotic cells (1, 29). The analysis presented here shows that uracil excision is not required to produce the strand specificity signal. The same conclusion was reached very recently for strand-specific mismatch repair in *S. pneumoniae* (4). Whether nicks on the newly replicated strand are produced by another mechanism or by any of a number of mechanisms, each of which is dispensable in the presence of the other(s), remains unanswered. The answer to these type of questions may come from the study of mismatch repair itself and the *PMS* genes participating in mismatch repair in *S. cerevisiae*.

ACKNOWLEDGMENTS

This work was supported in part by grant GM32431 from the National Institutes of Health and by award 33086 from the Mallinckrodt Foundation. P.M.J.B. is an Established Investigator of the American Heart Association (860129).

REFERENCES

1. **Aprelikova, O. N., V. M. Golubovskaya, I. A. Kusmin, and N. V. Tomilin.** 1989. Changes in the size of pulse-labelled DNA fragments induced in human cells by inhibitors of uracil-DNA-glycosylase and DNA methylation. Mutat. Res. **213**:135–140.
2. **Boeke, J. D., J. Truehart, G. Natsoulis, and G. R. Fink.** 1987. 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154**:164–175.
3. **Burgers, P. M. J., and M. B. Klein.** 1986. Selection by genetic transformation of a *Saccharomyces cerevisiae* mutant defective for the nuclear uracil-DNA-glycosylase. J. Bacteriol. **166**:905–913.
4. **Chen, J.-D., and S. A. Lacks.** 1991. Role of uracil-DNA-glycosylase in mutation avoidance by *Streptococcus pneumoniae*. J. Bacteriol. **173**:283–290.
5. **Claverys, J.-P., and S. A. Lacks.** 1986. Heteroduplex deoxyribonucleic acid base mismatch repair in bacteria. Microbiol. Rev. **50**:133–165.
6. **Drake, J. W.** 1991. A constant rate of mutation in DNA based microbes. Proc. Natl. Acad. Sci. USA **88**:7160–7164.
7. **Fix, D. F., and B. W. Glickman.** 1987. Asymmetric cytosine deamination revealed by spontaneous mutational specificity in an Ung$^-$ strain of *Escherichia coli*. Mol. Gen. Genet. **209**:78–82.
8. **Frederico, L. A., T. A. Kunkel, and B. R. Shaw.** 1990. A sensitive genetic assay for the detection of cytosine deamination: determination of rate constants and the activation energy. Biochemistry **29**:2532–2537.
9. **Giroux, C. N., J. R. A. Mis, M. K. Pierce, S. E. Kohalm, and B. A. Kunz.** 1988. DNA sequence analysis of spontaneous mutations in the *SUP4*-o gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **8**:978–981.
10. **Grafstrom, R. H., B. Y. Tseng, and M. Goulian.** 1978. The incorporation of uracil into animal cell DNA *in vitro*. Cell **15**:131–140.
11. **Grilley, M., J. Holmes, B. Yashar, and P. Modrich.** 1990. Mechanisms of DNA mismatch correction. Mutat. Res. **236**: 253–267.
12. **Hay, R. T., and M. L. DePamphilis.** 1982. Initiation of SV40 DNA replication in vivo: location and structure of 5′ ends of DNA synthesized in the ori region. Cell **28**:767–779.
13. **Holmes, J., S. Clark, and P. Modrich.** 1990. Strand-specific mismatch correction in nuclear extracts of human and Drosophila melanogaster cell lines. Proc. Natl. Acad. Sci. USA **87**:5837–5841.

13a. **Impellizzeri, K. J., and P. M. J. Burgers.** Unpublished data.

14. **Knapp, G., J. S. Beckwith, P. F. Johnson, S. A. Fuhrman, and J. Abelson.** 1978. Transcription and processing of intervening sequences in yeast tRNA genes. Cell **14**:221–236.
15. **Kohalmi, S. E., and B. A. Kunz.** 1988. Role of neighboring bases and assessment of strand specificity in ethylmethanesulphonate and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine mutagenesis in the *SUP4*-o gene of *Saccharomyces cerevisiae*. J. Mol. Biol. **204**: 561–568.
16. **Kramer, B., W. Kramer, M. S. Williamson, and S. Fogel.** 1989. Heteroduplex DNA correction in *Saccharomyces cerevisiae* is mismatch specific and requires functional *PMS* genes. Mol. Cell. Biol. **9**:4432–4440.
17. **Kramer, W., B. Kramer, M. S. Williamson, and S. Fogel.** 1989. Cloning and nucleotide sequence of DNA mismatch repair gene *PMS1* from *Saccharomyces cerevisiae*: homology of PMS1 to procaryotic MutL and HexB. J. Bacteriol. **171**:5339–5346.
18. **Kunkel, T. A.** 1985. The mutational specificity of DNA polymerases alpha and gamma during *in vitro* DNA synthesis. J. Biol. Chem. **260**:12866–12874.
19. **Kunz, B. A., L. Kohalmi, X. Kang, and K. A. Magnusson.** 1990. Specificity of the mutator effect caused by disruption of the *RAD1* excision repair gene of *Saccharomyces cerevisiae*. J. Bacteriol. **172**:3009–3014.
20. **Lahue, R. S., K. G. Au, and P. Modrich.** 1989. DNA-mismatch correction in a defined system. Science **245**:160–164.
21. **Langle-Rouault, F., G. Maenhaut-Michel, and M. Radman.** 1987. GATC sequences, DNA nicks and the mutH function in *Escherichia coli* mismatch repair. EMBO J. **6**:1121–1127.
22. **Lindahl, T., and B. Nyberg.** 1974. Heat induced deamination of cytosine residues in deoxyribonucleic acid. Biochemistry **13**: 3405–3410.
23. **Modrich, P.** 1989. Methyl-directed DNA mismatch correction. J. Biol. Chem. **264**:6597–6600.
24. **Muster-Nassal, C., and R. Kolodner.** 1986. Mismatch correction catalyzed by cell-free extracts of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **83**:7618–7622.
25. **Percival, K. J., M. B. Klein, and P. M. J. Burgers.** 1989. Molecular cloning and primary structure of the uracil-DNA-glycosylase gene from *Saccharomyces cerevisiae*. J. Biol. Chem. **264**:2593–2598.
26. **Pierce, M. K., C. N. Giroux, and B. A. Kunz.** 1987. Development of a yeast system to assay mutational specificity. Mutat. Res. **182**:65–74.
27. **Schaaper, R. M., and R. L. Dunn.** 1987. Spectra of spontaneous mutations in *Escherichia coli* strains defective in mismatch correction: the nature of *in vivo* DNA replication errors. Proc. Natl. Acad. Sci. USA **84**:6220–6224.
28. **Struhl, K., D. T. Stinchcomb, S. Scherer, and R. W. Davis.** 1979. High-frequency transformation of yeast: autonomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA **76**:1035–1039.
29. **Tomilin, N. V., and O. N. Aplrelikova.** 1989. Uracil-DNA-glycosylases and DNA uracil repair. Int. Rev. Cytol. **114**:125–179.
30. **Vollberg, T. M., K. M. Siegler, B. L. Cool, and M. A. Sirover.** 1989. Isolation and characterization of the human uracil-DNA-glycosylase gene. Proc. Natl. Acad. Sci. USA **86**:8693–8697.