Mutagenic specificity of endogenously generated abasic sites in *Saccharomyces cerevisiae* chromosomal DNA

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Abasic [apurinic/aprimidinic (AP)] sites are common, noncoding DNA lesions. Despite extensive investigation, the mutational pattern they provoke in eukaryotic cells remains unresolved. We constructed *Saccharomyces cerevisiae* strains in which chromosomal AP sites were generated during normal cell growth by altered human uracil-DNA glycosylases that remove undamaged cytosines or thymines. The mutation target was the *URA3* gene inserted near the *ARS309* origin to allow defined replication polarity. Expression of the altered glycosylases caused a 7- to 18-fold mutator effect in AP endonuclease-deficient (Δ *apn1*) yeast, which depended highly on the known translesion synthesis enzymes Rev1 and DNA polymerase ζ . For the C-glycosylase, GC>CG transversions were the predominant mutations, followed by GC>AT transitions. AT>CG transversions predominated for the T-glycosylase. These results support a major role for Rev1-dependent dCMP insertion across from AP sites and a lesser role for dAMP insertion. Unexpectedly, there was also a significant proportion of dTMP insertions that suggest another mutational pathway at AP sites. Although replication polarity did not strongly influence mutagenesis at AP sites, for certain mutation types, there was a surprisingly strong difference between the transcribed and non-transcribed strands of *URA3*. The basis for this strand discrimination requires further exploration.

Abasic [apurinic/aprimidinic (AP)] sites in DNA are very common lesions that arise directly from spontaneous base hydrolysis and free-radical reactions or indirectly through base damage caused by diverse endogenous and exogenous agents (1). The mutagenic effects of unrepaired AP sites result from DNA synthesis on these damaged templates (2). The normal replicative DNA polymerases cannot readily copy past AP sites (2), which, instead, require a specialized class of enzymes, the translesion synthesis (TLS) enzymes (for a review, see ref. 3). The presumptive evolutionary advantage of TLS enzymes is to allow cell survival of otherwise lethal DNA damage. Because AP sites lack the biochemical information required to identify the correct base, TLS enzymes may insert a nucleotide that results in a mutation.

The known TLS-related proteins in *Saccharomyces cerevisiae*, Rev1, Rev3, Rev7, Pol32, and Rad30, have been characterized genetically and biochemically. Yeast Rev1 acts *in vitro* as a deoxycytidyl transferase, inserting dCMP opposite lesions (4). Rev1 also has a modulatory role for other TLS enzymes (5). Pol32 is a nonessential subunit of the major yeast replicative DNA polymerase δ that promotes insertion of, predominantly, dAMP opposite AP sites *in vitro* (6). These insertions are extended by the postmismatch DNA polymerase ζ , a heterodimer of Rev3 and Rev7 proteins (5). Rad30 encodes DNA polymerase η , which, *in vitro*, inserts mainly dGMP or dAMP opposite AP sites (7), although recent *in vivo* studies do not support such a role (8, 9). After extension, synthesis switches back to normal DNA-replication enzymes (10, 11).

In a study using the yeast *SUP4-o* system, the most dramatic mutational effect of *Apn1*-deficiency was the generation of AT>CG transversions (12). These mutations depended partly on

the Mag1 3-methyladenine-DNA glycosylase, which led to the suggestion that such transversions might represent preferential dGMP insertion opposite the AP site. This interpretation rested on a narrow view of Mag1 specificity, and later work showed preferential insertion of dCMP during DNA synthesis past AP sites on plasmids transformed into yeast (13). Yeast transformation with AP-site-containing oligonucleotides (14) also suggested a "C rule" for insertion by TLS enzymes. A recent study indicates that spontaneous AP sites in *S. cerevisiae* are due mostly to excision of uracil incorporated opposite adenine (15). That observation may account for the AT>CG transversions found with *SUP4-o* (12), although it does not explain the partial *MAG1* dependence of those mutations.

Examination of mutagenesis in cells treated with DNA-damaging agents leads to somewhat different conclusions. Mutagenesis in yeast exposed to methyl methanesulfonate (which produces numerous AP sites) suggested preferential insertion of dAMP, consistent with an "A rule" for repair of abasic sites (5). A significant limitation of this interpretation, however, is that most of the observed mutations involved GC base pairs, where dCMP incorporation opposite a G-derived AP site would restore the wild-type sequence. Thus, uncertainties about the identity of the mutagenic AP sites attend both traditional studies of spontaneous mutation and studies of mutation induced by DNA-damaging agents.

Although the approach of using plasmids or oligonucleotides with known AP sites or glycosylase targets has been informative, it is uncertain whether DNA replication after introduction into the cells accurately reflects the processes acting on chromosomal genes. We have now overcome these limitations by employing modified versions of human uracil-DNA glycosylase (UDG) that remove either normal cytosines or normal thymines from yeast nuclear DNA (16), coupled to a high-throughput approach that allows all of the main classes of mutations to be identified at statistically significant levels.

Materials and Methods

Strains and Plasmids. Most of the yeast strains used here were derived from strain BGY111 (*MATa*, *his3- Δ* , *leu2*, *ura3-52*, *trp1-289a*, *can1*, and *GAL⁺*) (17). BGY111 was modified by transformation with pNKY1009, resulting in a *trp1- Δ 1::hisG* genotype (18), to generate strain LBY2. LBY2 strains carrying a deletion of *APN1* were constructed by transformation with pSCP19A (19), carrying an *apn1- Δ 1::HIS3* module, to create the strain LBY2a. Strains

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Abbreviations: AP, Abasic (apurinic/aprimidinic); CDG, cytosine-DNA glycosylase; CM-leu, leucine-deficient complete minimal medium; ODG, oxanine-DNA glycosylase; TDG, thymine-DNA-glycosylase; TLS, translesion synthesis; UDG, uracil-DNA-glycosylase; 5-FOA, 5-fluoroorotic acid.

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Fig. 1. Mutation rates in yeast expressing the CDG and TDG glycosylases. The upper part of the figure shows the structure of the *URA3* mutation targets inserted at the *ADP1* locus in strains LB20a and LB21a. Mutation rates to *ura3* were estimated by selecting for resistance to 5-FOA, as described in the text.

LB20 and LB20a were further modified by introducing *URA3* as a disruption of the *ADP1* gene. The nonessential *ADP1* gene (20) is located on chromosome III ≈ 1.7 kb from the 5' end of the early firing *ARS309* replication origin (21) (Fig. 1). Control experiments showed that this disruption did not affect the spontaneous mutation rate (to Can^R; data not shown). Construction of the DNA used for the disruption of the *ADP1* gene with *URA3* inserted in both orientations is provided in *Supporting Text*, which is published as supporting information on the PNAS web site.

These manipulations yielded strains LB20 (*APN1*) and LB20a (*apn1-Δ1::HIS3*) with *URA3* transcriptional orientation as for *ADP1* and LB21 (*APN1*) and LB21a (*apn1-Δ1::HIS3*) with *URA3* transcriptional orientation opposite *ADP1* (Fig. 1). The *ADP1::URA3* disruption had no detectable effect on the cell growth rate or cellular sensitivity to various mutagens (UV light, methyl methanesulfonate, and hydrogen peroxide).

Strain FY69 (*APN1*, *URA3*) was provided by Fred Winston of the Department of Genetics at Harvard Medical School (Boston).

Construction of *rev1* and *rev7* Knockout Strains. Knockout strains were constructed by using the protocols of the *Saccharomyces* Genome Deletion Project (http://sequence-www.stanford.edu/group/yeast_deletion_project). BY4741 yeast strains with the *REV1* or *REV7* locus replaced with the G418 (geneticin)-resistance cassette *kanMX4* were purchased from American Type Culture Collection, and the cassettes with flanking genomic sequences were amplified by PCR. The resulting PCR fragments were used to transform strain LB20a. After selection by G418 resistance, gene replacement was confirmed by PCR. The transformants were further screened for increased sensitivity to killing by 254-nm UV light (22). The *rev1::kanMX4* strain was designated PAY01a, and the *rev7::kanMX4* strain was designated PAY07a.

Construction of Glycosylase Expression Vector. Derivatives of the human uracil glycosylase gene encoding mutant enzymes that excise normal thymines or cytosines from DNA are described in ref. 16. Excision of the glycosylase genes from the original vectors and ligation into plasmid pYES2 is detailed in *Supporting Text*. The plasmids produced were pODG, the empty vector control; pCDG, encoding the cytosine-excising enzyme; and pTDG, encoding the thymine-excising enzyme (16).

Glycosylase expression was controlled by the *GAL1* promoter. For routine growth, strains bearing the glycosylase expression plasmids were cultured in leucine-deficient complete minimal medium (CM-leu) (23) with glucose to maintain the plasmid and

inhibit glycosylase expression. For glycosylase expression, strains were transferred to CM-leu with raffinose to clear the cells of glucose and then to CM-leu containing galactose (cells from the raffinose cultures seeded at $\approx 3,000$ cells per ml) and the cultures incubated as described below in *Identification and Sequencing of *Ura3*⁻ Mutants*.

Western Blot Analysis. Liquid cultures for LB20a and LB21a strains containing pODG, pCDG, or pTDG were grown to saturation during a 2–3 day incubation in CM-leu containing glucose, raffinose, or galactose. Cultures containing galactose were seeded from cells grown in raffinose, as described above. Protein extracts of these cultures were prepared under denaturing conditions (24). Western blot analysis was performed as described in the Fig. 4, which is published as supporting information on the PNAS web site.

Identification and Sequencing of *Ura3*⁻ Mutants. Strains carrying putative mutations in *URA3* were selected on medium containing 5-fluoro-orotic acid (5-FOA, Research Products International, Mt. Prospect, IL) (25). The 5-FOA-resistant colonies were then re-streaked onto plates containing complete minimal medium lacking uracil (CM-ura) to confirm the expected uracil auxotrophy. The 5-FOA-resistant colonies unable to grow on CM-ura plates were inoculated into 1-ml CM-leu plus glucose. After the cultures reached saturation (≈ 2 days), the cells were collected and genomic DNA extracted by using a kit (Epicentre, Madison, WI), and the inserted *URA3* locus was amplified by PCR. The PCR conditions were an initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 0.5 min, 56°C for 0.5 min, 72°C for 2.5 min, and a final extension step at 72°C for 7 min. Samples containing a PCR product with size corresponding to the full *URA3* gene (≈ 1.3 kb) were then purified (Qiagen, Valencia, CA), sequenced, and the results catalogued. The DNA sequencing was performed at the Dana–Farber/Harvard Cancer Center High-Throughput DNA Sequencing Facility.

Determination of Mutation Rates. Mutation rates were determined by using the method of the median (26). Briefly, 20 5-ml cultures were first grown to saturation, as described above, in CM-leu plus galactose. Three samples from each culture tube were plated onto 5-FOA plates and three diluted (1:10,000) samples onto yeast extract/bacto-peptone/dextrose (YPD) plates. The mean number of colonies on the YPD plates was divided into the corresponding total number of 5-FOA^R colonies to give a mutation frequency for each culture. The median value of the 20 mutation frequencies was then used to estimate the mutation rate from a statistical table (26).

Categorization of Orientation Differences. Mutations were scored in relation to the *URA3* coding sequence (nontranscribed strand), so that, in the case of cytosine-DNA-glycosylase (CDG)-induced mutants, a substituted cytosine indicates that the mutation originated from a base removed in the nontranscribed strand, whereas a substituted guanine indicated that the mutation originated from a base removed in the transcribed strand. Thymine-DNA-glycosylase (TDG)-induced mutants were scored analogously. As shown in Fig. 1 (Top), in the LB20 background, *URA3* is expressed codirectionally with leading-strand DNA synthesis, with *URA3* in the LB21 background having the opposite orientation.

Statistical Analyses. Differences in the frequencies of strand-specific base substitutions were analyzed for significance by a comparison of proportions or χ^2 analysis. In each case, a *P* value < 0.05 indicated a significant difference and a rejection of the null hypothesis (that there were no strand-specific differences in base substitutions).

Results

Mutagenesis Driven by Mutant UDGs. The mutagenic effects of AP sites formed by the modified glycosylases (CDG and TDG) in *S. cerevisiae* were explored initially in three strain backgrounds: LB20

Table 3. Base substitution frequencies

Strain	Transitions			Transversions				
	Total	AT>GC	GC>AT	Total	AT>TA	AT>CG	GC>TA	GC>CG
LB20a ODG	12	4 (5)	8 (9)	73	8 (9)	18 (22)	40 (47)	7 (8)
LB21a ODG	7	2 (4.5)	5 (11)	37	3 (7)	7 (16)	25 (57)	2 (4.5)
LB20a CDG	57	1 (0.5)	56 (27)	154	3 (1.5)	6 (3)	41 (19)	104 (49)
LB21a CDG	24	0	24 (26)	67	0	5 (6)	18 (20)	44 (48)
LB20 CDG	10	0	10 (24)	31	0	6 (15)	18 (44)	7 (17)
LB20a TDG	8	6 (3)	2 (1)	231	36 (15)	178 (74)	16 (7)	1 (<1)
LB21a TDG	7	4 (3)	3 (2)	135	20 (14)	105 (74)	8 (6)	2 (1)
LB20 TDG	4	0	4 (9)	40	5 (11)	17 (39)	17 (39)	1 (2)

Data in parentheses are percentages.

were distributed at both A·T and G·C base pairs, with some preference for the latter (Table 3). When the CDG enzyme was expressed in the *apn1-Δ1* background, predominantly GC>CG transversions were observed, ≈50% of all single-base-pair substitutions. Also observed at high frequency with CDG were GC>AT transitions (≈26%), and GC>TA transversions (≈20%). Expression of the TDG enzyme in the *apn1-Δ1* background showed mostly AT>CG (74%) and AT>TA (≈15%) transversions (Table 3).

CDG-induced mutants in the *APN1* strain LB20 yielded a similar frequency of GC>AT transitions as in the *apn1-Δ1* background (24% vs. 26–27%) but different frequencies of GC>TA transversions (≈44% vs. 19–20%) and GC>CG transversions (≈17% vs. 48–49%) (Table 3). In the TDG-induced mutants, AT>CG transversions made up ≈39% of observed mutations in the *APN1* strain, compared with ≈74% in the *apn1-Δ1* background, and AT>TA transversions made up ≈11% of observed mutations, similar to the ≈15% in the *apn1-Δ1* strains (Table 3).

An *APN1* strain (FY69) with *URA3* at the normal chromosomal locus was also studied to determine how the location of *URA3* might influence the overall spectrum of mutations. CDG-induced mutations in FY69 had similar proportions compared with LB20 of GC>AT transitions (16% vs. 24%) and GC>TA transversions (36% vs. 44%) but a higher proportion of GC>CG transversions (39% vs. 19%; $z = 2.30$ and $P < 0.05$).

Mutations in *APN1* strains expressing the TDG enzyme did not show the same degree of targeting as found for expression of CDG in these strains or of TDG expressed in the *apn1-Δ1* strains. However, the overall TDG-induced mutation patterns in FY69 (data not shown) and LB20 (Table 3) were quite similar, indicating that there are no differences in the local environment between *URA3* and *ADP1::URA3* that significantly influence the mutagenic processing of T-derived AP sites.

To determine which classes of mutations were most influenced by the mutant glycosylases, we calculated how the rate of each type of single-base-pair substitution changed with expression of each glycosylase. Mutation rates were determined by normalizing the frequency of each mutation type as a function of the overall mutation rate for each strain. The overall patterns of distribution of the normalized mutation rates within each strain showed some differences between the LB20a orientation and the LB21a orientation. Both GC>CG and GC>TA transversions driven by CDG occurred at approximately three times the rate in LB20a (Fig. 2A) compared with LB21a (Fig. 2B). CDG-driven GC>AT transitions were also higher in LB20a compared with LB21a (Fig. 2). In contrast, there was much less replication-orientation difference for the TDG-driven mutations (Fig. 2), especially when corrected for an AT>CG hotspot (see below and see Fig. 3).

The physical distribution of mutations in the *URA3* gene is shown in Fig. 3. The LB20a and LB21a orientations are directly compared in each strain, with the central axis line representing the entire *URA3* sequence. For ODG, spontaneous mutations (Fig. 3A) were found somewhat more frequently in the first half of the *URA3* gene. The CDG-induced mutations (Fig. 3B) were scattered a little more

evenly throughout the gene. For CDG-driven mutagenesis, there were a few positions frequently mutated in both *URA3* orientations at base pairs 22, 89, 181, 286, 344, 461, and 550. TDG-induced mutations in the *apn1-Δ1*, *URA3::ADP1* background (Fig. 3C) showed a generally similar pattern for both orientations, including a pronounced AT>CG transversion hotspot at position 449. In contrast, a TDG-generated hotspot at position 398 in LB20a was much less pronounced in the LB21a orientation (Fig. 3C). TDG expression also generated a relatively high frequency of AT>CG mutations at position 449 in the *APN1*⁺ strains LB20 and FY69 (data not shown). The sequence around this hotspot (CTTTT-GATGTTAGCAGAATTG; the italicized T represents the hotspot at position 449) does not provide an obvious explanation for the targeting of mutations at this position.

Effect of Gene Orientation on Distribution of Single-Base-Pair Substitutions. To determine whether replication polarity influences AP-site mutagenesis, we examined the types of base substitutions in each strand of both orientations of the *URA3* target (see *Materials and Methods*). Overall, in both LB20a and LB21a cells, the replication orientation had rather little effect. However, in both orien-

Fig. 2. Rates of individual base-substitution mutations. The data from Tables 2 and 3 were used with the overall mutation rates (Fig. 1) to estimate mutation-specific values. For the AT>CG column, a value is also shown with the AT>CG hotspot at position 449 (see Fig. 3C) subtracted.

frequent, is consistent with previous studies in *S. cerevisiae* (9, 13, 14, 37), confirming results obtained from distinctly different experimental methods. Insertion of dGMP occurred rarely in all studies where it could be established unambiguously.

The most unusual category of point mutations provoked by AP sites are GC>TA and AT>TA transversions seen in response to CDG and TDG, respectively (Table 3). Such mutations could arise from insertion of dTMP opposite the AP site during mutagenic DNA synthesis, an activity that has not been associated with yeast DNA polymerases. There is evidence that Rev1 modulates dTMP insertion independently of its dCMP transferase activity (14), which may indicate that another enzyme or modifying activity is yet to be found. Alternatively, some aspect of DNA or chromosome structure *in vivo* allows dTMP insertion that has not been observed *in vitro*. Analysis of the genetic dependence of specific mutation subtypes should shed some light on this intriguing observation.

Our results with the Rev1- and Pol- ζ -deficient strains showed several informative effects. The *rev1 Δ* allele lowered the basal mutation rate 13-fold relative to the *REV*⁺ strain (Table 1), consistent with published observations (17). Although CDG and TDG elevated this rate $\approx$ 8-fold and 3-fold, respectively, the rates were still far below those observed in *REV*⁺ cells expressing the glycosylases, and the apparent increases in mutation rates were not statistically significant. Thus, mutagenesis by AP sites in yeast depends heavily on *REV1*, but there is still a small *REV1*-independent component. The residual CDG-induced mutagenesis in the *rev1 Δ* strain could include the GC>AT A-rule substitutions, because this class of mutation is the most frequent in Rev1-deficient strains (38), but such a simple picture would predict, based on Table 3, a higher CDG-induced mutation rate than we observed. The CDG-induced GC>TA and TDG-induced AT>TA transversions could also be formed in a Rev1-independent fashion but, again, would seem to be making a rather minor contribution in the *rev1 Δ* strain. In the case of the *rev7 Δ* strain, mutagenesis in response to CDG or TDG was virtually completely suppressed (values not significantly different from ODG), although there may be a slight mutagenic trend in response to TDG (Table 1). However, Rev7-deficiency did not have nearly the effect on the basal mutation rate as did Rev1-deficiency. Surprisingly, the 290-bp deletions produced by gene conversion evidently also strongly depend on *REV1* and *REV7*. Because gene conversions constituted $\approx$ 40% of the total mutations in LB20a (Table 2), one would expect a much higher residual mutation rate in *rev1 Δ* and *rev7 Δ* expressing CDG or TDG, if the gene-conversion events were independent of the TLS en-

zymes. The mechanism of the apparent TLS-dependence of gene conversion induced by endogenous AP-site formation is unclear, but will be interesting to explore.

We were surprised to find only modest effects of the replication orientation on mutation rates or specificity in the *URA3* reporter gene (Fig. 1; Table 4). Others have reported strand specificity for spontaneous mutations that appear to be due to polymerase errors (39, 40). Evidently, the processes in operation at AP lesions in yeast differ from those that give rise to strand-specificity in replication errors. Whereas, in *E. coli*, replication polarity affects replicative (41, 42) and damaged-induced (43) mutagenesis differently, the situation in yeast has been unresolved, and our results shed light on one type of DNA damage.

There was, however, a very strong bias for TDG-induced mutations that depended on the transcriptional orientation (Table 4). TDG-induced transversions were nearly 10-fold more frequent for the nontranscribed than for the transcribed strand. Such a difference could arise from an unknown Apn1-independent repair mechanism that operates preferentially on the transcribed strand, for example. However, such a bias was not observed for CDG-driven mutations, which suggests transcribed-strand-specific processing of AP sites opposite A, or discrimination in the TDG glycosylase itself. In the absence of Apn1, AP sites could become more susceptible to AP lyases, such as Ntg1 and Ogg1. However, these enzymes cleave AP sites preferentially opposite pyrimidines (44) or opposite C nearly exclusively (45), so their involvement would seem to require AP/C and AP/T sites generated by replication past the AP site. The mutator glycosylase properties also do not provide a ready explanation: TDG is about equally effective on single-stranded compared with double-stranded DNA, whereas CDG has some preference for single-stranded DNA substrates (16).

A refined analysis of AP site mutagenesis supports the proposed critical roles of some TLS proteins, notably Rev1 and Rev7. Some mutation types, and the unexpected effects of transcription polarity, indicate that significant mutational pathways remain to be analyzed. With such effects detectable, even with the transient expression of the mutator DNA glycosylases, it will be of interest to know whether more severe or chronic accumulation of abasic DNA damage changes the contributions of novel mutational pathways.

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