Specificity of the mutator caused by deletion of the yeast structural gene (APN\textsuperscript{I}) for the major apurinic endonuclease

((basic sites)/yeast SUP\textsuperscript{4-o} gene/Escherichia coli lacZ gene/endonuclease IV/exonuclease III))

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ABSTRACT The loss of bases from cellular DNA occurs via both spontaneous and mutagen-induced reactions. The resulting apurinic/apyrimidinic (AP) sites are cytotoxic and mutagenic but are counteracted by repair initiated by AP endonucleases. Previously, in vitro and bacterial transfection studies suggested that AP sites often prompt insertion of dAMP residues during replication, the A-rule. Dissimilar results have been obtained by transfecting DNA into eukaryotic cells. It seemed possible that these differences might be due to idiosyncratic sites of transfection or aberrant replication of the transfected DNA. The observation that AP endonuclease-deficient strains of the yeast Saccharomyces cerevisiae have elevated spontaneous mutation rates allowed us to determine the mutational specificity of endogenously generated AP sites in nuclear DNA. With the yeast SUP\textsuperscript{4-o} gene as a mutational target, we found that a deficiency in the major yeast AP endonuclease, Apn1, provoked mainly single base-pair substitution; the rate of transposon Ty insertion was also enhanced. The rate of transversion to a G-C pair was increased 10-fold in Apn1-deficient yeast, including a 59-fold increase in the rate of A-T \rightarrow C-G events. In contrast, the rate of transversion to an A-T pair was increased by only 3-fold. A deficiency in N\textsuperscript{3}-methyladenine glycosylase offset these substitution rate increases, indicating that they are due primarily to AP sites resulting from glycosylase action. Thus, the A-rule does not seem to apply to the mutagenic processing of endogenous abasic sites in S. cerevisiae. Other results presented here show that AP endonuclease-deficient Escherichia coli exhibit a mutator phenotype consistent with the A-rule.

DNA repair synthesis and ligation (4). AP endonucleases and the genes that encode them have been isolated from bacteria, the yeast Saccharomyces cerevisiae, Drosophila melanogaster, and mammalian cells (5-11). In Escherichia coli, exonuclease III and exonuclease IV account for \(\approx 95\%\) of the total AP endonuclease activity in cell extracts (5, 6, 8). The biological role of the E. coli enzymes has been confirmed by the observation that enzyme-deficient strains are hyper-sensitive to and hypermutable by agents that generate abundant AP sites, such as DNA-methylating compounds. Analogous experiments (12) demonstrate the in vivo function of Apn1, the major AP endonuclease of S. cerevisiae. Moreover, Apn1-deficient strains of yeast exhibit a substantially elevated rate of spontaneous mutation for several test genes and for different conditions (12). These data suggest that unrepaired AP sites produced endogenously are indeed mutagenic lesions.

The mutational specificity of AP sites was first explored in transfection experiments in which single-stranded bacteriophage DNA was introduced into E. coli, with the specific changes established by analysis of the DNA recovered from the cells (13, 14). In these experiments, the mutations found were mainly transversions that apparently resulted from the insertion of dAMP residues opposite the abasic sites during replication. Analysis of the template specificity of AP sites during in vitro DNA synthesis by a variety of DNA polymerases has indicated a preference for insertion of dAMP (15-20). These and other results gave rise to the so-called A-rule (noninstructional insertion of adenine by DNA polymerase), which has since been applied to explain the mutational specificity of a variety of DNA lesions that lack obvious coding capacity (21). Subsequent experiments involving transfection of AP site-containing DNA into eukaryotic cells suggested that the mutational specificity of AP sites might be different in nucleated cells (22-24). However, transfection experiments can be criticized at least on the grounds that the incoming DNA might undergo additional modification or be subject to a type of DNA synthesis that does not represent the actual chromosomal replication mechanism. Given the mutator effect of Apn1 deficiency in yeast (12), we chose to examine this issue for spontaneously produced AP sites in the nucleus of S. cerevisiae. The results indicate that unrepaired endogenous AP sites have significant mutagenic potential in eukaryotes, but with a mutational specificity different from that expected on the basis of the A-rule.

Abbreviations: BPS, base-pair substitution; SBPS, single base-pair substitution; AP, apurinic/apyrimidinic.
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MATERIALS AND METHODS

Strains, Plasmid, and Media. The haploid, repair-proficient yeast strain MKP-op (MATa, can1-100, ade2-1, lys2-1, ura3-52, leu2-3,112, his3-A2000, trpl-A901, YCpmP2) (25) and isogenic derivatives deleted either for APNI (apnl-ΔI:: HIS3) (DRY373-p) (12) or APNI and MAG (N'-methyleneamino glycosylase) (apnl-ΔI::HIS3, mag::LEU2) (WXMA-p) (12, 26) were used. SUP4-o, an ochre suppressor allele of a yeast tyrosine tRNA gene, is carried on YCpmP2 (25), a yeast centromere-containing vector. Such plasmids mimic chromosome behavior and are maintained primarily as single copies in haploid yeast cells (27). Isogenic derivatives of E. coli CC101-CC110 (28) lacking exonuclease III (Δxth) and endonuclease IV (Δpol::kan) were constructed by transduction of the two repair mutations from strains BW9116 (Δxth- pncA90, zdh-201::TnlO) and BW527 (Δpol::kan). Strain JF1754 (25) was also used. Media for growth of yeast or bacteria and selection of SUP4-o mutants or lacZ+ revertants have been described (28, 29).

Detection of Spontaneous Mutations. Forward mutations in SUP4-o were selected by scoring for reduced suppression of the ochre markers can1-100, ade2-1, and lys2-1 (25). This procedure detects at least a 30% decrease in functional SUP4-o tRNA (30) and does not bias mutant recovery significantly (31, 32). lacZ+ revertants were selected by selecting for the Lac+ phenotype (28).

Mutant Isolation, Plasmid Retention, Mutation Frequency and Rate. SUP4-o mutations were collected and plasmid retention and mutation frequencies were determined as described (25). SUP4-o mutation rates were calculated according to the formula (33) \( \mu = (0.4343Cfm)/\log(Np) \), where \( \mu \) = mutations per DNA replication, \( C = \) reciprocal of efficiency of base-pair substitution (BPS) detection, calculated as [no. of non-BPSs detected + [no. of BPSs detected × (no. of possible BPSs)/(no. of detectable BPSs)])/no. of mutations sequenced [of 267 possible BPSs in SUP4-o, 178 are detectable (32)], \( f_m \) = median mutation frequency (MKP-op, 2.3 \times 10^{-6}; DRY373-p, 8.9 \times 10^{-6}; WXMA-p, 1.3 \times 10^{-6}), and \( N = \) median final population size (MKP-op, 3.92 \times 10^{6}; DRY373-p, 1.53 \times 10^{6}; WXMA-p, 3.83 \times 10^{3}) including three generations on the selection plates (determined microscopically) before nonmutants cease growing. To isolate lacZ+ revertants, E. coli strains were diluted in LB medium to \( \sim 1000 \) cells/ml, grown overnight at 37°C, and plated on minimal lactose or minimal glucose medium for Lac+ revertants or viable cells, respectively (28). Plates were scored after 3 days incubation at 37°C.

Other Methods. YCpmP2 was released from yeast cells by disruption with glass beads (34), transformed into JF1754 by using calcium chloride (25), and isolated from JF1754 by an alkaline extraction procedure (35). SUP4-o alleles were sequenced on double-stranded YCPmP2 molecules (36). \( x^2 \) tests with Yates’ correction for continuity (37) were used to assess differences in several parameters. The null hypothesis that the apnl-ΔI mutator did not influence the distribution of substitutions in SUP4-o was evaluated by the Monte Carlo estimate of the \( P \) value of the hypergeometric test (38). For both tests, values of \( P < 0.05 \) were considered significant.

RESULTS

Plasmid Stability and Spontaneous Mutation Rate in Yeast. MKP-op (APNI) and DRY373-p (apnl-ΔI) were grown aerobically from low-titer inocula to stationary phase in medium selective for the plasmid. The cultures were then plated to measure plasmid retention and to select canavanine-resistant colonies. Since the two strains are isogenic except for the APNI locus, any differences here been due to the apnl-ΔI mutator effect and not to variation in genetic back-
spontaneous DNA alkylation was largely responsible for the SBPS specificity of the \textit{apnl-\Delta l} mutator. Thus, it is interesting that the frequency of A-T \to C-G transversions induced by DNA ethylation in hamster cells was markedly decreased by simultaneous treatment with methoxamine (39), which specifically reacts with abasic lesions.

The distributions of all the SBPSs recovered in the wild-type and \textit{apnl-\Delta l} strains are compared in Fig. 1. Changes were found only at positions in the \textit{SUP4-o} exons, with the exception of the intronic site 51. Altogether, substitutions were detected at 62 different sites; of these, 35 positions were mutated in both distributions. Mutations specific to MKP-op or DRY373-p were found at 13 or 14 sites, respectively. At 34 of the 35 common positions, substitution rates were elevated in DRY373-p with a range of 1.3- to 14-fold, with a mean increase of 4.5-fold. There was little overlap in the sites mutated most frequently, and the A-T \to C-G transversion hot spots at sites 30 and 87 in the \textit{apnl-\Delta l} distribution were especially notable in this regard. Although such transversions can be detected at 23 separate positions in \textit{SUP4-o} (32), the 54 A-T \to C-G substitutions recovered were located on only 13 different sites. These observations suggest that local DNA sequence context modulates the specificity of the \textit{apnl-\Delta l} mutator. Application of a statistical test (38) to compare the two distributions indicated that this putative site specificity was not due to random sampling error (\( P < 0.002 \)).

\textbf{Specificity of \textit{lacZ} Reversion in \textit{\Delta xth nfo::kan E. coli Strains}.} The finding that defective repair of endogenous AP sites in yeast provokes a predominant increase in the rate of transversion to G-C base pairs contrasts with expectations based on the study of AP site mutagenesis in \textit{E. coli} (see Introduction). To our surprise, we were unable to identify any published report on the possible mutational specificity of AP endonuclease deficiency in \textit{E. coli}. We therefore constructed a series of test strains defective in both the \textit{xth} (encoding exonuclease III) and \textit{nfo} (encoding endonuclease IV) genes. These enzymes together constitute \( \approx \)95% of the total AP endonuclease activity in growing \textit{E. coli} (5, 6, 8). Null mutations were introduced into a series of strains (28) that can be used to detect individually all 6 SBPSs at one site within \textit{lacZ} by scoring for reversion to the Lac\(^+\) phenotype. The AP endonuclease deficiency had a spontaneous mutator effect that varied with the \textit{lacZ} allele examined (Table 4). In contrast to the results for yeast, however, the largest frequency increase (22-fold) was seen for A-T \to T-A transversions, while the frequency of A-T \to C-G transversions increased by only 3-fold. It should be noted, however, that results for reversion at single sites (as in the \textit{lacZ} system) must be generalized with caution; the positions in question might coincidentally be hot or cold spots for specific SBPSs induced by particular lesions.

\section*{DISCUSSION}

The frequent and potentially mutagenic loss of DNA bases (3) is counteracted by AP endonucleases, enzymes that are

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Sites} & \textbf{Changes} & \textbf{No. detected} & \textbf{APNI} & \textbf{apnl-\Delta l} \\
\hline
5, 6 \to 7 & G \to A, -1 & 2 & - & - \\
6 \to 7 & -1 & 1 & - & - \\
6 \leftrightarrow 7 & Ty & 1 & - & - \\
14 \to -890* & -904 & - & 1 & - \\
17 \leftrightarrow 18 & Ty & 1 & - & 1 \\
18 \leftrightarrow 19 & Ty & 1 & - & 1 \\
21 \leftrightarrow 22 & Ty & 1 & - & 1 \\
23 \leftrightarrow 24 & -1 & - & 2 & - \\
24 & -1 & 1 & - & - \\
25 \to 26 & -1 & - & 2 & - \\
36, 43 & A \to C, T \to G & - & 1 & - \\
35, 36 & A \to G, A \to G & 1 & - & - \\
37 \leftrightarrow 38 & Ty & 11 & 11 & - \\
46 \to -106† & -152 & 2 & - & - \\
51, 52, 53 & C \to A, T \to G, T \to C & 1 & - & - \\
55 \to -559‡ & -614 & 1 & - & - \\
58 \to 64 & -7, +GGGCCC & 3 & - & - \\
74 & -1 & 1 & - & - \\
79 \to 83 & -1 & 22 & 7 & - \\
79 \to 83 & +1 & 4 & 4 & - \\
79 \to 83, 84 & +1, C \to A & - & 1 & - \\
87, 88 & T \to C, C \to T & - & 1 & - \\
88 \to 96§ & -9 & 2 & - & - \\
126 \to -107 & - & 232 & - & - \\
\hline
\end{tabular}
\caption{Multiple mutations, deletions, and insertions}
\end{table}

The \textit{APNI} and \textit{apnl-\Delta l} strains were examined for the \textit{SBPSs} that were detected in the \textit{xth nfo::kan E. coli} strains (28). The results are shown in Table 3. The distribution of the SBPSs was compared with the distribution in the \textit{apnl-\Delta l} strain. The most striking deviation was observed for the \textit{SBPSs} at site 30, which is a hot spot for AU mismatch repair in yeast. The frequency of this \textit{SBPS} was reduced in the \textit{apnl-\Delta l} strain, indicative of a decrease in the rate of repair by mismatch repair in yeast. The distribution of the \textit{SBPSs} in the \textit{apnl-\Delta l} strain was also compared with the distribution in the \textit{APNI} strain. The results are shown in Table 4. The frequency of the \textit{SBPSs} at site 87 was increased in the \textit{APNI} strain, indicative of a decrease in the rate of repair by mismatch repair in yeast. The distribution of the \textit{SBPSs} in the \textit{APNI} strain was also compared with the distribution in the \textit{apnl-\Delta l} strain. The results are shown in Table 5. The frequency of the \textit{SBPSs} at site 87 was increased in the \textit{APNI} strain, indicative of a decrease in the rate of repair by mismatch repair in yeast.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
\textbf{Substitution} & \textbf{APNI} & \textbf{Rate, \( \times 10^{-8} \)} & \textbf{apnl-\Delta l} & \textbf{Rate, \( \times 10^{-8} \)} & \textbf{apnl-\Delta l magA} & \textbf{Rate, \( \times 10^{-8} \)} \\
\hline
G-C \to A-T & 44 & 25.3 & 10.6 & 19 & 11.5 & 20.7 & 13 & 19.7 & 7.5 \\
A-T \to G-C & 27 & 15.5 & 6.5 & 16 & 9.7 & 17.4 & 7 & 10.6 & 4.1 \\
Total & 71 & 40.8 & 17.1 & 35 & 21.2 & 38.1 & 20 & 30.3 & 11.6 \\
G-C \to T-A & 58 & 33.4 & 14.0 & 31 & 18.8 & 33.8 & 9 & 13.6 & 5.2 \\
A-T \to T-A & 7 & 4.0 & 1.7 & 11 & 6.7 & 12.0 & 4 & 6.1 & 2.3 \\
G-C \to C-G & 34 & 19.5 & 8.2 & 34 & 20.6 & 37.2 & 16 & 24.2 & 9.3 \\
A-T \to C-G & 4 & 2.3 & 1.0 & 54 & 32.7 & 58.9 & 17 & 25.8 & 9.8 \\
Total & 103 & 59.2 & 24.9 & 130 & 78.8 & 141.8 & 46 & 69.7 & 26.6 \\
\hline
\end{tabular}
\caption{SBPSs}
\end{table}

For the \textit{apnl-\Delta l magA} strain, 67 mutations were characterized, and one was a base-pair insertion.
ubiquitous (5). Elimination of the main such enzyme (Apn1) from S. cerevisiae confers a mutator effect (12), the specificity of which we have established in this study. The spontaneous mutator spectrum is dominated by transversions to G-C base pairs, which account for more than half of the increase in the mutation rate. In contrast, transversions to A-T base pairs may highlight the mutator phenotype of AP endonuclease-deficient E. coli.

We have obtained evidence that abasic sites are critical targets for Apn1 in its role to limit the rate of spontaneous mutagenesis in yeast. The mutator effect resulting from Apn1 deficiency was strongly offset by the introduction of a second mutation causing a deficiency in the MAG-encoded N3-methyladenine glycosylase. This enzyme extracts N3- and N7-methylated purines (26) to produce AP sites but may have a broader substrate specificity that includes nonalkylation damages such as 8-hydroxyguanine (41), an oxidized base. Consistent with a possible role for oxidative damage in the apn1-Δmutator effect, the rate of spontaneous mutation was lowered when an apn1-Δ strain was grown anaerobically, but the mutator phenotype was still evident under those conditions (12). This outcome is consistent with both spontaneous oxidation and alklylation contributing to the formation of AP sites during aerobic growth. Thus, our results, plus a similar finding for reversion of the trp1-289 allele (26), indicate that spontaneous purine alkylations in the yeast genome are processed by the N3-methyladenine glycosylase to AP sites that become targets for mutagenesis in the absence of Apn1. Although Apn1 also removes deoxyribose fragments at free radical-induced strand breaks (5), mutagenesis by such damage has not been reported.

Table 4. lacZ reversion in Δxth nfo::kan E. coli strains

<table>
<thead>
<tr>
<th>BPS</th>
<th>Mutation frequency, × 10^{-10}</th>
<th>Relative increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-C → A-T</td>
<td>216 ± 44</td>
<td>0.7</td>
</tr>
<tr>
<td>A-T → G-C</td>
<td>2.0 ± 1.5</td>
<td>6.6</td>
</tr>
<tr>
<td>G-C → T-A</td>
<td>136 ± 35</td>
<td>4.4</td>
</tr>
<tr>
<td>A-T → T-A</td>
<td>17.8 ± 7.2</td>
<td>22.0</td>
</tr>
<tr>
<td>G-C → C-G</td>
<td>3.5 ± 3.4</td>
<td>0.9</td>
</tr>
<tr>
<td>A-T → C-G</td>
<td>13 ± 7.3</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Frequencies are means ± SD for four independent experiments. Relative increase = (Δxth nfo::kan)/wild type.

During normal aerobic growth, AP sites probably are also produced in yeast in at least two other ways: (i) by glycosylase-mediated excision of uracil from DNA (42); and (ii) by spontaneous oxidative damages (besides 8-hydroxyguanine) that either directly cause abasic sites or give rise to modified bases (e.g., thymine glycol) that are substrates for glycosylases other than the MAG gene product (5). However, the dramatic effect of N3-methyladenine glycosylase deficiency on the apn1-Δl mutator suggests that abasic sites created by the MAG-encoded enzyme may outnumber those generated by other means. AP sites from these other sources likely contribute to the residual levels of mutagenesis detected in the apn1-Δl magΔ strain.

Previously, the strength of the apn1-Δl mutator was found to vary at different genetic loci (12), but a large increase in the rate of transversion to G-C base pairs was not predicted. Our data show that most of these changes are due to loss of DNA purines. Consequently, our findings suggest that AP sites in yeast trigger the insertion of dGMP during translesion DNA synthesis. This preference does not arise from a selection bias in the mutational assay system used, because in SUP4-o the insertion of dAMP opposite purines can be detected at more sites than insertion of dGMP (63 vs. 52 sites, respectively) (32).

Numerous published studies indicate a significant tendency of both prokaryotic and eukaryotic DNA polymerases to insert dAMP rather than dGMP opposite AP sites in vitro (3, 13–21). Some nonphysiological aspect of these systems (the presence or absence of auxiliary replication factors, the lack of native chromatin structure, etc.) could underlie this in vitro partiality. Alternatively, the apparent preference for dGMP insertion might be the result of a pre- or postreplication step. Perhaps unrepaired AP sites in yeast can react with some other cellular component to generate lesions with modified mutational properties. For example, the aldehyde form of deoxyribose has the potential to form a Schiff's base with protein lysines, although this reaction is expected to be slow (2). A postreplication repair system also could process duplex AP site-containing intermediates but in yeast fail to act on the AP:dGMP structure. Such a repair mechanism might be considered analogous to the MutY glycosylase that removes adenine inserted opposite 8-hydroxyguanine during DNA replication in E. coli (43). Of course, our experiments cannot rule out the possibility that a mutagenic process, which acts on lesions other than AP sites, is triggered in

Fig. 1. Distribution of SBPSs in SUP4-o. Only the region of the transcribed strand encoding the tRNA is shown. The anticodon is at 36–38 and the 14-bp intron is inferred to extend from 40 to 53 as for the sup4+ allele (40).
that such patterns do not result from the activation of an antimutagenic system that dampens mutagenesis at the lesions responsible for spontaneous mutation in the wild-type strain.

The reversion pattern we observed for lacZ missense alleles in AP endonuclease-deficient E. coli is more consistent with previous studies suggesting a preference for DAMP insertion opposite AP sites in this organism. For several reasons, we cannot conclude that the different specificities of the yeast and E. coli mutators necessarily point to dissimilarities in the processing of endogenous AP sites. First, different mutational targets and assays were used (SUP4-o forward system for yeast vs. lacZ reversion system for E. coli), making any comparison rather speculative. Substitution rates at individual sites depend on the incidence and processing of damage, factors that probably are influenced by the context of flanking sequences. Second, it is not certain that the specificity of the AP endonuclease-deficient E. coli mutator reflects a failure to repair only AP sites. Both exonuclease III and endonuclease IV also act on other DNA lesions that might have contributed to the mutational pattern observed in the bacterial strain. Third, AP site mutagenesis requires activation of the SOS system in E. coli (3, 13). Although this response can be provoked by the presence of unrepaired AP sites (44), the extent to which SOS may have been induced in the AP endonuclease-deficient strain was not determined.

Finally, the apnl-Δ1 mutator also increased the rate of insertion of the yeast retrotransposon Ty into SUP4-o. Elsewhere, we have suggested that transposition of Ty might be activated by spontaneous DNA damage (45). Since deletion of APN1 diminishes repair of AP sites and free radical-induced strand breaks, our results indicate that failure to eliminate specific, spontaneously occurring DNA lesions can promote Ty transposition.

Although this and many previous studies (3) support the operation of an A-rule in E. coli, transfection experiments with a site-specifically located abasic site in double-stranded DNA suggested the lack of such a rule in rodent and simian cells (23, 24). While such transfection studies have not been reported for S. cerevisiae, our results and spontaneous mutagenesis in Apnl-deficient yeast suggest that mutagenic processing of AP sites might also differ in this eukaryote. Taken together with the mammalian cell studies, these observations argue that the A-rule cannot be readily extrapolated to nucleated cells.

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