Cellular Role of Yeast Apn1 Apurinic Endonuclease/3'-Diesterase: Repair of Oxidative and Alkylation DNA Damage and Control of Spontaneous Mutation

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The *APN1* gene of *Saccharomyces cerevisiae* encodes the major apurinic/apyrimidinic endonuclease and 3'-repair DNA diesterase in yeast cell extracts. The Apn1 protein is a homolog of *Escherichia coli* endonuclease IV, which functions in the repair of some oxidative and alkylation damages in that organism. We show here that yeast strains lacking Apn1 (generated by targeted gene disruption or deletion-replacement) are hypersensitive to both oxidative (hydrogen peroxide and *t*-butylhydroperoxide) and alkylating (methyl- and ethylmethane sulfonate) agents that damage DNA. These cellular hypersensitivities are correlated with the accumulation of unrepaired damages in the chromosomal DNA of *apn1* mutant yeast cells. Hydrogen peroxide-treated *APN1*⁺ but not *apn1* mutant cells regenerate high-molecular-weight DNA efficiently after the treatment. The DNA strand breaks that accumulate in the Apn1-deficient mutant contain lesions that block the action of DNA polymerase but can be removed in vitro by purified Apn1. An analogous result with DNA from methylmethane sulfonate-treated cells corresponded to the accumulation of unrepaired DNA apurinic sites in the *apn1* mutant cells. The rate of spontaneous mutation in *apn1* mutant *S. cerevisiae* was 6- to 12-fold higher than that measured for wild-type yeast cells. This increase indicates that under normal growth conditions, the production of DNA damages that are targets for Apn1 is substantial and that such lesions can be mutagenic when left unrepaired.

Cellular DNA damage arises from hydrolytic reactions, endogenous reactive agents such as ·OH, or exposure to many exogenous agents. Spontaneous depurination and depyrimidination may amount to ~10⁴ events per day in human cells (17) accompanied by additional damage to both purines and pyrimidines in DNA (35). The hydroxyl radical is probably the proximal species that causes DNA damage by oxidative mutagens (11) and accounts for much of the cellular damage caused by ionizing radiation (35). The anti-tumor drugs bleomycin (6, 26) and neocarzinostatin (24) also mediate free-radical attack on DNA and cause some of the same DNA damages produced by X rays and other oxidative mutagens. These lesions include oxidized abasic sites and DNA strand breaks (10, 35). The latter bear 3' fragments of deoxyribose that have to be removed to allow DNA repair synthesis (3, 8, 27). Apurinic/apyrimidinic (AP) sites are also generated indirectly by alkylation of DNA, which forms modified bases that readily undergo hydrolysis of their glycosylic bonds either spontaneously or via DNA glycosylases (19).

AP sites in DNA are targets for mutagenesis because they lack template information, so that DNA polymerases incorporate nucleotides (often dAMP) which may not be appropriate (20). The repair of AP sites in DNA is initiated by AP endonucleases, the main class of which (the so-called class II AP endonucleases) hydrolyzes the phosphodiester bond immediately 5' to the abasic site (16, 20). In bacteria, these class II AP endonucleases also remove the 3'-terminal fragments borne by the DNA strand breaks that arise from free-radical attack (3, 27). Such blocking groups, if left

unrepaired, might also block DNA synthesis to induce the SOS response, which is an error-prone DNA repair pathway (36).

Enzymes that attack AP sites in DNA and restore primers for DNA polymerase have been isolated from both prokaryotic and eukaryotic sources (37, 38). The major AP endonuclease-3'-repair diesterase from the yeast *Saccharomyces cerevisiae* has been characterized biochemically (12, 13), and the structural gene (*APN1*) encoding this enzyme was cloned and sequenced (23). The predicted Apn1 protein is homologous to DNA endonuclease IV of *Escherichia coli* (23). In keeping with this homology, Apn1 protein can substitute for the DNA repair functions of its bacterial counterpart, endonuclease IV (27). Yeast haploid strains with an *apn1-1::URA3* disruption were drastically defective in both AP endonuclease and DNA repair 3'-diesterase activities (23), confirming that these functions reside in a single protein. In the present work, we examine the physiological role of the Apn1 protein in yeast cells and directly demonstrate that the enzyme repairs oxidative and alkylation damages to yeast chromosomal DNA in vivo. This in vivo function extends to a role for Apn1 in limiting spontaneous mutagenesis in yeast cells.

MATERIALS AND METHODS

Strains, plasmids, and media. The *S. cerevisiae* strains used in this study were DBY747 (*APN1*⁺) and SCP762 (*apn1-1::URA3*) as described previously (23); SCP762-F1 (*apn1-1::hisG*), a Ura⁻ derivative of SCP762 selected with 5-fluoroorotic acid (31); MKP-o (*MATα can1-100 ade2-1 lys2-1 ura3-52 leu2-3,112 his3-Δ200 trp1-Δ901*), kindly provided by B. Kunz, University of Manitoba, Winnipeg, Manitoba, Canada (22); and DRY370 and DRY373, each carrying the *apn1-Δ1::HIS3* allele (see below) and isogenic

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to DBY747 and MKP-o, respectively. The plasmids used were YEp351 (9) and YEpAPN1 (23). Cells were grown in either complete YPD medium or minimal synthetic (SD) medium (31). Nutritional supplements (20 μ g/ml) were added as required.

Construction of a Δ *apn1* strain. Plasmid pSCP19 containing a 2.9-kb *EcoRI*-*EcoRV* DNA fragment that bears the entire *APN1* gene and flanking yeast sequences was derived from pSCP8 (23) by *EcoRI* and *EcoRV* partial digestion. The *APN1* translational start codon is ~1.4 kb from the *EcoRV* end of the 2.9-kb fragment (23). The plasmid pSCP19 was digested with *StuI* and *SnaBI* to remove 751 bp of the *APN1* coding region (bp 353 to 1131, which includes the stop codon at bp 1102 to 1104; numbering as in reference 23), recircularized by ligation, and subsequently digested with *BglIII*, which cuts the *APN1* fragment at bp 22. The resulting linear plasmid was ligated to a 1.45-kb *BamHI* fragment containing the *S. cerevisiae* *HIS3⁺* gene (33) to yield plasmid pSCP19A. A linear 3.2-kb fragment (*apn1*- Δ 1::HIS3) was obtained from pSCP19A by digestion with *EcoRI* and *BamHI* and used to transform the yeast strains DBY747 and MKP-o to yield, respectively, strains DRY370 and DRY373. The chromosomal deletion-replacement was verified in these strains by Southern blot analysis (data not shown) and by activity measurements (see Results).

Purification and assay of Apn1. The yeast Apn1 endonuclease was purified according to the published procedure (12) with minor modifications. Briefly, EDTA and dithiothreitol were omitted from all buffers, and protease inhibitors were added (aprotinin, leupeptin, and pepstatin, each to 1 μ g/ml; benzamide and phenylmethylsulfonyl fluoride, each to 1 mM). These changes increased the stability and yield of the enzyme and eliminated the requirement for a metal cofactor for activity. Crude extracts of yeast cells were prepared and assayed as previously described (14, 23).

Survival curves. The sensitivity of the yeast strains to either H₂O₂ or *t*-butylhydroperoxide was measured by using exponential-phase cultures. Overnight cultures grown to saturation at 30°C in YPD were diluted into fresh YPD to an optical density at 600 nm (OD₆₀₀) of 0.2 (~2 $\times$ 10⁶ cells per ml) and incubated at 30°C to an OD₆₀₀ of 0.8 to 1.0. Aliquots of those cultures were then treated with the indicated concentrations of H₂O₂ (Fluka Chemicals) or *t*-butylhydroperoxide (Sigma Chemical Co.) at 30°C with shaking (200 rpm) for 60 min. Surviving cells were determined by diluting the samples in sterile distilled water and plating them onto YPD plates that contained 2% agar. Colonies were counted after 2 to 3 days of growth at 30°C.

Treatment of yeast cells with ethylmethane sulfonate (EMS; Sigma) or methylmethane sulfonate (MMS; Aldrich) was done by using a modification of the procedure of Prakash and Prakash (25). Briefly, cells from an overnight culture (~5 $\times$ 10⁷ cells per ml) were pelleted, washed once in sterile distilled water, washed in sterile 50 mM potassium phosphate buffer (pH 7.5), and then resuspended in a volume of the same buffer equal to the original culture volume. The cell suspensions were then treated with the indicated concentrations of EMS or MMS for 1 h at 30°C with shaking. Viability was determined as described above.

Sucrose gradients. Exponentially growing cells in SD medium containing 1 μ g of uracil per ml were labeled with [6-³H]uracil (17 Ci/mmol; New England Nuclear) as described by Reynolds (28). H₂O₂ treatment (20 mM H₂O₂ for 1 h at 30°C) was stopped by the direct addition of bovine catalase (Sigma) to the culture to a final level of 50 U/ml. The incubation was then continued in this medium for 10 min or

in YPD medium for 5 h, as indicated in Results. In the case of MMS, cells were treated with 0.15% MMS for 1 h at 30°C and, for posttreatment incubation, washed twice with 1 ml of sterile distilled water prior to incubation at 30°C in YPD medium. After incubation, the cells were prepared for alkaline sucrose density gradient sedimentation according to the method described by Reynolds (28), except that the gradients were spun in a Beckman SW56Ti rotor at 35,000 rpm for 130 min. The gradients were calibrated with ³H-labeled T7 DNA (molecular weight, 2.5 $\times$ 10⁷), which was kindly provided by Raymond Kim, Harvard University.

Primer activation experiments. Overnight cultures of DBY747 (*APN1⁺*) and DRY370 (*apn1*- Δ 1::HIS3) were diluted into 10 ml of fresh YPD to an OD₆₀₀ of 0.25. The cultures were incubated at 30°C with shaking (300 rpm) until the OD₆₀₀ was ~0.9. Treatment of the cells with various agents was done as described above. Treated cells were subsequently collected by centrifugation and washed twice with 10 ml of distilled water prior to DNA isolation. The DNA was isolated according to the method described by Rose (29), except that the pipette tips were truncated to avoid shearing of the chromosomal DNA. The yield of DNA from 10 ml of culture was 5 to 10 μ g.

Primer activation reactions contained 1 μ g of yeast chromosomal DNA in a 25- μ l reaction mixture containing 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH (pH 7.5), 200 mM KCl, 100 μ g of bovine serum albumin per ml, and, where indicated, 0.4 U of purified yeast Apn1 protein. The reactions were incubated at 37°C for 30 min and then heated at 70°C for 3 min to inactivate the Apn1 protein. These samples were chilled on ice, and 225 μ l of chilled DNA polymerase I reaction mix was added (25 mM HEPES-KOH [pH 7.6]; 25 mM KCl; 50 μ g of bovine serum albumin; 10 mM MgCl₂; 100 μ M each dATP, dCTP, and dGTP; [methyl-³H]dTTP [91 Ci/mmol, Amersham] at a specific activity of 1,260 cpm/pmol). The tubes were transferred to a 37°C water bath, DNA polymerase I (0.4 U; Bethesda Research Laboratories) was added, and, at the indicated times, 40- μ l aliquots of samples were withdrawn to prechilled tubes on ice. The samples were precipitated with trichloroacetic acid to determine the incorporation of [methyl-³H]dTTP (3).

Spontaneous mutation rates. The spontaneous mutation rates were determined by using the fluctuation test method described by von Borstel (34). For each test, at least 24 1-ml cultures were grown in 24-well tissue culture dishes (Costar). Reversion of *ade2-1* or *lys2-1* (ochre mutations) or *trp1-289* (amber mutation) was measured in cultures grown in SD medium (31) limited in adenine (0.75 μ g/ml), lysine (1.0 μ g/ml), or tryptophan (1.25 μ g/ml). Other required amino acids were supplied in excess. Under these conditions, the initial cell titer was ~5,000 cells per ml, and the final cell density in unrevoked wells was ~1.0 $\times$ 10⁶ cells per ml; reversion events allowed much denser growth. For anaerobic experiments, anaerobic and control media were supplemented with 0.1% Tween 80 and 30 μ g of ergosterol per ml. The BBL GasPak system was used for anaerobic growth. The mutation rate was calculated from the final cell density in unrevoked wells and the number of wells with no mutations by using the Poisson distribution.

RESULTS

Sensitivity of *apn1* mutants to oxidants and alkylating agents. Yeast strains bearing either a disruption in the *APN1* gene (*apn1*-1::URA3) or a deletion-replacement (*apn1*- Δ 1::

TABLE 1. AP endonuclease and 3'-diesterase activities in crude extracts of various yeast strains

Strain	Plasmid	Sp act in crude extract (U/mg of protein) ^a	
		AP endo-nuclease	3'-Diesterase
DBY747 (<i>APN1</i> ⁺)	None	56	1.8
DBY747	YEp351	52	2.4
DBY747	YEpAPN1	980	36.0
DRY370 (<i>apn1-Δ1::HIS3</i>)	None	<1	<0.08
DRY370	YEpAPN1	972	41.0
SCP762 (<i>apn1-1::URA3</i>)	None	<1	<0.05
MKP-o (<i>APN1</i> ⁺)	None	58	1.6
DRY373 (<i>apn1-Δ1::HIS3</i>)	None	<1	<0.07

^a Activities were determined in crude extracts of cells grown either in YPD medium or in the appropriate SD medium for plasmid-containing strains.

HIS3) are nearly devoid of both 3'-repair diesterase and AP endonuclease activities (23) (Table 1). We hypothesized that such strains might be hypersensitive to H₂O₂, since DNA damages generated in vivo by this agent in *E. coli* include deoxyribose fragments with blocked 3' termini at DNA strand breaks (3). The AP endonuclease deficiency of the mutant strains also suggested that they would be hypersensitive to alkylating agents such as MMS and EMS, which lead to the formation of AP sites (4).

The *apn1-Δ1::HIS3* or *apn1-1::URA3* allele in haploid yeast cells did cause hypersensitivity not only to H₂O₂ but also to the organic peroxide *t*-butylhydroperoxide (Fig. 1A and B). The *apn1* mutant strains were also considerably more sensitive to the killing effects of the alkylating agents MMS and EMS (Fig. 1C and D). Using the data in Fig. 1, we calculate that DRY370 (*apn1-Δ1::HIS3*) was 10-, 2.5-, 7-, and 4-fold more sensitive to, respectively, H₂O₂, *t*-butylhydroperoxide, MMS, and EMS than the wild-type parental strain DBY747 (comparison of 50% lethal dose for each agent). Similar sensitivities were also seen when strain

FIG. 1. Sensitivity of *S. cerevisiae* strains to oxidative and alkylating agents. Symbols: ○, *APN1*⁺ strain (DBY747); ●, *apn1-Δ1::HIS3* strain (DRY370). t-BH, *t*-butylhydroperoxide.

FIG. 2. Alkaline sucrose density gradient analysis of DNA damage in the chromosomes of H₂O₂-treated yeast cells. Symbols: ○, *APN1*⁺ strain (DBY747); ●, *apn1-Δ1::HIS3* strain (DRY370). (A) Untreated; (B) treated with 20 mM H₂O₂ for 1 h; (C) as for panel B and incubated posttreatment for 5 h. Vertical arrows indicate the sedimentation positions of bacteriophage T7 DNA. TCA, trichloroacetic acid.

SCP762 was compared with DBY747 (data not shown). However, the *apn1* mutant strains were not more sensitive than wild-type yeast cells to gamma rays, bleomycin, mitomycin C, paraquat, menadione, 4-nitro-quinoline-1-oxide, or 254-nm UV light (data not shown).

Accumulation of H₂O₂ damages in vivo in *apn1* mutant strains. We wished to test whether the mutagen sensitivity of *apn1* mutant yeast cells could be correlated with damages in their chromosomal DNA. Hydrogen peroxide produces DNA strand breaks as a direct damage (11, 35), and these breaks can be detected readily by sedimentation analysis (3). Sedimentation in alkaline sucrose gradients of chromosomal DNA from yeast cells immediately after a challenge with H₂O₂ revealed a higher level of DNA strand breakage in an *apn1* mutant than in its wild-type parent (Fig. 2). However, when the cells were incubated in peroxide-free growth medium following the H₂O₂ treatment, DNA of nearly native size was re-formed after 5 h in the wild-type strain. A much lesser extent of rejoining of oxidative strand breaks was observed in the *apn1* mutant. Thus, the single-strand breaks resulting from H₂O₂ damage require the Apn1 enzyme for their efficient repair. In addition to strand breaks, oxidatively produced abasic sites could contribute to the DNA fragmentation seen in alkaline sucrose gradients. However, such base-free sites are not major products in H₂O₂-treated DNA (3, 11), and their contribution was not determined separately.

FIG. 3. Primer activation assay of H_2O_2 damage in yeast chromosomal DNA in vivo. Open symbols, samples not pretreated in vitro with purified Apn1; filled symbols, samples treated in vitro with purified Apn1. (A and B) DNA from cells treated with 20 mM H_2O_2 for 1 h; (C and D) DNA from cells treated with H_2O_2 and allowed a recovery period of 5 h; (A and C) *APN1*⁺ strain (DBY747); (B and D) *apn1*⁻ strain (DRY370). Triangles, samples from cells not treated with a damaging agent; circles, DNA from damaged cells.

The structure of the H_2O_2 -induced DNA strand breaks that persist in Apn1-deficient yeast strains was assessed by using an enzymatic probe, *E. coli* DNA polymerase I. This enzyme requires normal DNA 3'-hydroxyl termini but is blocked for synthesis on chromosomal DNA from exonuclease III-deficient *E. coli* (*xth* mutant) treated with H_2O_2 (3). The Apn1 protein activates these primers for DNA polymerase both in vitro (13) and in vivo (27) by restoring normal 3'-hydroxyl termini. We tested whether analogous blocked 3' termini accumulate in the chromosomes of Apn1-deficient yeast cells.

Low levels of DNA synthesis were observed with DNA samples from unchallenged wild-type or *apn1* mutant cells (Fig. 3A and B). However, when the cells were challenged with 20 mM H_2O_2 , the dTMP incorporation (Fig. 3A) increased 17-fold in the DNA isolated from the parent strain (~10% survival) and only 3-fold with samples from an *apn1* mutant strain (~0.05% survival). To assess whether DNA isolated from H_2O_2 -treated cells contained additional Apn1-sensitive sites, the damaged DNA was pretreated with purified Apn1 in vitro prior to the DNA polymerase incubation. This pretreatment stimulated dTMP incorporation in DNA isolated from H_2O_2 -challenged *apn1* mutant strains by approximately sevenfold (Fig. 3B) while producing no detectable increase in synthesis with damaged DNA from wild-type cells (Fig. 3A). The range of in vitro primer activation for DNA from *apn1* mutant cells was 7- to 12-fold, while no significant effect of pretreatment with Apn1 was seen with DNA from any undamaged cells over many experiments. A similar analysis was carried out with H_2O_2 -treated cells that were allowed to recover for 5 h in growth medium after oxidative damage. The additional incubation was given to allow time for the damaged cells to repair their DNA in vivo. Under these conditions, the extent of dTMP

FIG. 4. Alkaline sucrose density gradient analysis of DNA damage in the chromosomes of MMS-treated yeast. Symbols: ○, *APN1*⁺ strain (DBY747); ●, *apn1*⁻ strain (DRY370). (A) Untreated; (B) treated with 0.15% MMS for 1 h; (C) treated with MMS and incubated posttreatment for 5 h. Vertical arrows indicate the sedimentation positions of bacteriophage T7 DNA. TCA, trichloroacetic acid.

incorporation into DNA significantly decreased in the parent strain, unlike incorporation in the samples isolated directly after the H_2O_2 challenge (cf. Fig. 3C and A). The DNA from the recovered parent was not affected by pretreatment with Apn1 (Fig. 3C). In contrast, the amount of dTMP incorporation into DNA was not decreased if H_2O_2 -treated *APN1*⁺ cells were incubated for 5 h in 20 mM potassium phosphate buffer, pH 6.5, instead of growth medium (data not shown). The extent of DNA synthesis and activation by Apn1 samples from the *apn1* mutant strain did not change appreciably during the recovery period in growth medium (Fig. 3B and D). Evidently, the enzyme-deficient cells challenged with H_2O_2 accumulate DNA targets for Apn1 that persist for hours after the challenge but are completely removed from the chromosomes of wild-type cells during the time of the H_2O_2 treatment.

Accumulation of MMS-induced damage in *apn1* mutant cells. The hypersensitivities of *apn1* mutant strains to EMS and MMS suggested that these strains may accumulate abasic sites (AP sites) as a result of alkylation damage. Because AP sites are the only alkali-labile DNA damages produced by alkylating agents (4), the level of these lesions can also be assessed by sedimentation in alkaline sucrose density gradients. When yeast cells were treated with 0.15% MMS for 1 h, a higher level of strand breaks was observed in the *apn1* mutant than in the parent strain (Fig. 4B). This

FIG. 5. Primer activation assay of MMS damage in yeast chromosomal DNA in vivo. Open symbols, samples not pretreated in vitro with purified Apn1; filled symbols, samples treated in vitro with purified Apn1. (A and B) DNA from cells treated with 0.15% MMS for 1 h; (C and D) DNA from cells treated with MMS and allowed a recovery period of 5 h; (A and C) *APN1*⁺ strain (DBY747); (B and D) *apn1*⁻ strain (DRY370). Triangles, samples from undamaged cells; circles, DNA from damaged cells.

finding indicates the presence of a substantially greater number of AP sites in the mutant than in the wild-type strain. After a posttreatment (recovery) incubation for 5 h (Fig. 4C) or 8 h (not shown), the sedimentation rate (and therefore average size) of the DNA from both strains increased only slightly. The difference between the strains persisted, and even the wild-type yeast cells had not regenerated DNA of the size measured before damage. In order to define further the role of Apn1 protein in the repair of alkylation damage, primer activation experiments were performed with chromosomal DNA from cells treated with MMS. Abasic sites produced in vivo by either glycosylases or spontaneous hydrolysis can be attacked by Apn1 to form free 3'-OH for DNA repair synthesis (13, 14). A 10-fold increase in primer activity was seen with DNA obtained from *APN1*⁺ cells treated with MMS compared with the activity in undamaged cells (Fig. 5A). This primer activity could be stimulated a further threefold by a prior pretreatment in vitro with Apn1 (Fig. 5A). In contrast, the DNA from MMS-treated *apn1* mutant yeast cells exhibited no significant increase in primer activity but was activated to a level similar to that of the wild-type strain by purified Apn1 (Fig. 5B). After a recovery period of 5 h, the overall pattern persisted with both strains, except that the magnitude of DNA synthesis diminished (Fig. 5C and D). The apparent removal of some Apn1-sensitive sites during the posttreatment incubation of the *apn1* mutant strain (cf. Fig. 5B and D) is consistent with the partial restoration of higher-molecular-weight DNA during recovery of this strain (Fig. 4C).

Since some Apn1-sensitive sites persisted in the DNA of wild-type cells even after a recovery period, we hypothesized that Apn1 might be limiting for the repair of alkylation-induced AP sites. However, a nearly 20-fold overproduction of Apn1 in vivo (Table 1) did not further increase dTMP

FIG. 6. Primer activation assay of MMS damage in chromosomal DNA from yeast cells overexpressing Apn1. Open symbols, samples not pretreated in vitro with purified Apn1; filled symbols, samples treated in vitro with purified Apn1. DNAs were from the *APN1*⁺ strain (DBY747) (A) or the *apn1*⁻ strain (DRY370) (B) treated with 0.15% MMS for 1 h. Squares, DNA samples from the *APN1*⁺ strain (DBY747) (A) and the *apn1*⁻ strain (DRY370) (B) overproducing Apn1; circles, strains bearing the vector only.

incorporation into DNA from MMS-treated *APN1*⁺ cells (Fig. 6A), and significant primer activation by Apn1 in vitro was still observed (Fig. 6A). As expected, introduction of the *APN1*⁺ plasmid rendered the *apn1* mutant strain indistinguishable from its plasmid-containing wild-type parent with respect to the primer activation phenotype for MMS damage (Fig. 6B). Thus, the normal cellular level of Apn1 is apparently not limiting for the repair of alkylation damage in yeast cells.

As an alternative, it seemed possible that MMS could inactivate the Apn1 enzyme in vivo to account for the persistence of Apn1-sensitive sites in DNA from MMS-treated *APN1*⁺ cells. Cells treated with increasing concentrations of MMS did contain diminishing levels of AP endonuclease activity (Fig. 7), but the concentration of MMS (0.15%) used in our experiments reduced the level of active cellular Apn1 by only 30% (Fig. 7). Similarly, the concentration of H₂O₂ used in our experiments (20 mM) reduced the endogenous AP endonuclease activity by only 15%. Clearly, the slight inactivation of endogenous AP endonuclease by MMS cannot explain the presence of Apn1-sensitive sites in DNA isolated from MMS-damaged *APN1*⁺ cells. These sites could arise from AP sites that are inaccessible to repair in vivo or from residual methylated bases that spontaneously release during extraction and purification of the DNA.

Spontaneous mutation and Apn1. Both abasic sites (17, 20)

FIG. 7. Resistance of intracellular Apn1 to inactivation by H₂O₂ (A) or MMS (B). Crude extracts were prepared from an *APN1*⁺ strain (DBY747) grown in YPD medium and treated with increasing concentrations of either H₂O₂ or MMS for 1 h. The levels of class II AP endonuclease in the extracts were determined.

TABLE 2. Spontaneous mutation in *APN1*⁺ and *apn1* mutant *S. cerevisiae*

Strain	Mutation rate ^a /10 ⁸ cells/generation					
	Expt 1		Expt 2		Expt 3	
	Ade ⁺	Lys ⁺	Ade ⁺	Lys ⁺	Ade ⁺	Lys ⁺
MKP-o (<i>APN1</i> ⁺)	5.3 ± 1.3	6.2 ± 1.5	6.1 ± 1.6	4.2 ± 1.4	4.5 ± 0.7	7.2 ± 1.1
DRY373 (<i>apn1-Δ1::HIS3</i>)	46 ± 6.8	73 ± 9.6	36 ± 6.2	50 ± 7.9	39 ± 5.6	69 ± 8.8

^a Measured as described in Materials and Methods. The values shown are for three typical experiments from a total of six and reflect the ranges of values seen in all six experiments. Standard errors were calculated according to the formula used by von Borstel (34).

and oxidative damages (7, 32) can be formed under conditions prevalent in cells during normal growth. Thus, although Apn1 is not essential for the growth or survival of yeast cells under laboratory conditions, the enzyme could contribute to genetic stability. The possible role of Apn1 in spontaneous mutagenesis was therefore assessed in strain MKP-o (*APN1*⁺) and its isogenic *apn1* mutant counterpart, DRY373 (*apn1-Δ1::HIS3*). Both strains contain ochre mutations at two different loci (*ade2-1* and *lys2-1*) and require both adenine and lysine for growth. The spontaneous mutation rate was determined by using the fluctuation test (34) and scoring for Ade⁺ and Lys⁺ cells that appeared after limiting amounts of either adenine or lysine were exhausted. In the absence of Apn1 protein, the spontaneous mutation rate in this genetic background was increased by 6- to 9-fold for Ade⁺ and 10- to 12-fold for Lys⁺ (Table 2).

The selected Ade⁺ populations were dominated by suppressor mutants rather than true revertants: 26 wells from MKP-o and 27 wells from DRY373 yielded exclusively Ade⁺ Lys⁺ Can^s colonies. For the population selected as Lys⁺ in MKP-o, suppressors also seemed to dominate, as 24 wells produced exclusively Lys⁺ Ade⁺ Can^s cells. Only the *apn1-Δ1* strain seemed to produce true revertants, with 14 of 38 wells selected as Lys⁺ containing Lys⁺ Ade⁻ Can^r cells; the remainder contained Lys⁺ Ade⁺ Can^s cells. Using these data, we calculate that the rate of true reversion of the *lys2-1* mutation is increased more than 100-fold in Apn1-deficient cells compared with wild-type cells under these conditions.

In separate experiments in a different genetic background (DBY747), the *apn1-1::hisG* allele (in strain SCP762-F1) also increased the spontaneous mutation rate. In those experiments, the formation of Trp⁺ cells from a *trp1-289* amber mutant was scored and exhibited a 2.8-fold-higher rate in the *apn1* mutant background than in *APN1*⁺ (the rates [± standard deviation] were 9.8 ± 3.1 and 3.5 ± 1.1 per 10⁸ cells per generation, respectively). We do not know what fraction of the Trp⁺ cells arose from suppressors as opposed to true reversion mutations. It seemed possible that the increased rate of mutation seen in Apn1-deficient strains could arise from endogenously produced oxidative DNA damages (7). However, the presence of oxygen was not necessary to realize increased spontaneous mutagenesis in *apn1* mutant cells, since the anaerobically grown *apn1-1::hisG* strain still exhibited a 2.6-fold-higher mutation rate than that of *APN1*⁺ cells (the rates [± standard deviation] were 2.9 ± 0.9 and 1.1 ± 0.6 per 10⁸ cells per generation).

DISCUSSION

Yeast strains that lack the major 3'-diesterase/AP endonuclease (*apn1* mutants) are abnormally sensitive to both oxidative and alkylating agents. The multiple sensitivities of such strains support the idea that the Apn1 enzyme participates in multiple DNA repair pathways (13). In fact, the

hypersensitivity of Apn1-deficient yeast cells to both H₂O₂ and MMS is correlated with the persistence of chromosomal damages, specifically DNA strand breaks detectable by sedimentation in alkaline sucrose gradients and the enzyme-sensitive sites that yield DNA synthesis primers upon Apn1 treatment in vitro. The lesions formed by H₂O₂ remained virtually untouched in *apn1* mutant cells after removal of the agent but were removed in *APN1*⁺ cells. After MMS damage, the *apn1* mutant strain appeared to remove some lesions but at a much lower rate than was seen with the *APN1*⁺ strain. These findings together indicate that Apn1 protein is required in vivo for the efficient removal of 3'-terminal blocking lesions at DNA strand breaks and for the incision of AP sites. The multifunctional Apn1 enzyme is also involved in limiting the rate of spontaneous mutagenesis in yeast cells. This last observation is consistent with conclusions from other studies that both oxidative (7, 32) and alkylation (1, 30) damages to DNA are produced as by-products of normal growth.

The yeast Apn1 endonuclease shares both structural homology and biochemical specificity with endonuclease IV of *E. coli* (12, 13, 15, 23). The expression of yeast Apn1 endonuclease in *E. coli* conferred resistance on oxidants and alkylating agents in mutants lacking both endonuclease IV (*nfo* mutants) and exonuclease III (*xth* mutants) or in an *nfo* single mutant (27). However, yeast *apn1* mutant cells and *E. coli nfo* mutant cells do display phenotypic differences. We were surprised to find that the hypersensitivity of *nfo* mutant *E. coli* to bleomycin (2) was not reproduced in yeast *apn1* mutant strains. Perhaps the DNA damages produced by this agent can be repaired by an Apn1-independent pathway in yeast cells (e.g., recombinational repair [5]) but rely more heavily on endonuclease IV for repair in *E. coli*. Consistent with this suggestion, the bleomycin-sensitive yeast strains (21) we have tested all contained normal levels of Apn1 activities (unpublished data). It is certainly also possible that differences in cellular oxidative chemistry leading to differences in the damages caused could explain the requirement for endonuclease IV in *E. coli*, but not for Apn1 in yeast cells to counteract the toxicity of bleomycin.

The lack of increased sensitivity of yeast *apn1* mutant strains to bleomycin was also surprising because Apn1 is so quantitatively predominant in *S. cerevisiae*. Perhaps yeast cells harbor AP endonuclease or 3'-repair diesterase enzymes different from Apn1 that are poorly detected in our assays and that contribute particularly to the repair of some damages. Conceivably, the gene(s) encoding such enzymes could be identified in a biological test by screening for enhanced cellular resistance to some oxidants in *apn1* mutant cells bearing a multicopy plasmid library.

The increased spontaneous mutation rate in *apn1* mutant yeast cells is noteworthy because such an increase was not reported for similarly AP endonuclease-deficient *xth nfo*

double mutants of *E. coli* (2). Again, various explanations could account for this difference, depending on alternative damage-processing pathways or physiological chemistry in the two organisms. Such differences might affect the endogenous production of either oxidative or alkylating mutagens in yeast cells.

The strong effect of the *apn1-Δ1* mutation on reversion of the *lys2-1* allele is striking because the *ade2-1* allele did not seem to undergo reversion at a significant rate compared with the production of suppressor mutations in the same strains. This difference could reflect more-restricted possibilities for reversion of *ade2-1* than for *lys2-1*, but we have not found other information on this point. Alternatively, there could be differences in the reactivity of these two DNA sites with respect to endogenous mutagens that produce AP sites or oxidative damages.

The substrate specificity of *Apn1* and some known features of DNA chemistry suggest identities for possible spontaneous premutational lesions. AP sites are the best characterized of these and are expected to occur at a substantial rate under physiological conditions (18). AP sites in template DNA may provoke the insertion of dAMP during DNA replication (20), which results in transversion mutations at apurinic sites. In addition to their production by hydrolytic reactions in normal DNA (18), AP sites probably also arise as secondary products of endogenous DNA alkylation or oxidation (1, 7, 30, 32). Oxidative damage to DNA deoxyribose produces fragmented products in addition to abasic sites (35). It is not known whether these latter lesions can be processed to yield mutations. However, such sites could have indirect effects through their ability to block DNA replication, for example by triggering cellular pathways that increase mutagenic processing of other damages (5). It is clear that at least a portion of the increased spontaneous mutagenesis seen in *apn1* mutant yeast cells does not arise from oxidative damage, because such an increase was also seen in cells grown anaerobically.

We do not know whether spontaneous mutations that arise in wild-type cells are due to damages that could be repaired by *Apn1* but that escape repair for one reason or another. However, a detailed sequence analysis of the excess spontaneous mutations produced in *apn1* mutants, both aerobically and anaerobically, will shed light on the nature of the endogenous damages that *Apn1* ordinarily diverts to nonmutational pathways. That information in turn will help complete our picture of the important physiological DNA-damaging processes that evolution has coped with to attain genetic stability.

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