Molecular Mechanism of Base Excision Repair of Uracil-containing DNA in Yeast Cell-free Extracts*

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Base excision repair (BER) constitutes a ubiquitous excision repair mechanism, which is responsible for the removal of multiple types of damaged and inappropriate bases in DNA. We have employed a yeast cell-free system to examine the biochemical mechanism of the BER pathway in lower eukaryotes. Using uracil-containing DNA as a model substrate, we demonstrate that yeast BER requires Apn1 protein, an Escherichia coli endonuclease IV homolog. In extracts of an apn1 deletion mutant, the 5'-incision at AP (apurinic/apyrimidinic) sites is not detectable, supporting the notion that yeast contains only one major 5'-AP endonuclease. The processing of the 5'-deoxyribose phosphate moieties was found to be a rate-limiting step. During BER of uracil-containing DNA, repair patch sizes of 1–5 nucleotides were detected, with single nucleotide repair patches predominant.

Base excision repair (BER) is an important cellular process, which corrects multiple damaged and inappropriate bases in DNA. Substrates for BER include oxidative base damage, multiple forms of alkylation damage, AP (apurinic/apyrimidinic) sites formed by spontaneous loss of bases, and uracil residues in DNA. Studies in Escherichia coli and in human cells have demonstrated that BER of DNA is initiated by a class of enzymes called DNA glycosylases, which catalyze the hydrolysis of the N-glycosyl bond linking particular damaged bases to the sugar-phosphate backbone. Excision of free bases by this mechanism generates AP sites in the DNA (1, 2). The phosphodiester bonds 5' to these sites are then incised by an AP endonuclease, generating 3'-hydroxyl and 5'-deoxyribose phosphate moieties. The latter are removed by a DNA deoxyribophosphodiesterase (dRpase) activity, and the resulting single nucleotide gaps are filled in by a DNA polymerase. Covalent integrity of the DNA strands is finally restored by a DNA ligase (3).

DNA glycosylases can be divided into two classes: those without and with associated AP lyase activity (3). Uracil-DNA glycosylase and E. coli endonuclease III are examples of the former and the latter, respectively. The physiological significance of the associated AP lyase activity in BER remains an open question (3). In E. coli, exonuclease III and endonuclease IV constitute the majority of the 5'-AP endonuclease activity (3). A human counterpart of exonuclease III has been identified (4, 5). Several proteins have been found to possess dRpase activity, including formamidopyrimidine-DNA glycosylase, RecJ protein, and DNA polymerase β (6–8). DNA polymerase β is apparently the major enzyme that operates during repair synthesis of BER in higher organisms (9, 10), generating a single nucleotide repair patch (11, 12). Hence, its associated dRpase activity is probably also operative under normal conditions. Recent studies suggest that repair synthesis and DNA ligation in mammals may involve a protein complex containing DNA polymerase β, DNA ligase III, and XRCC1 protein (11). Human DNA ligase I has also been implicated in BER (13).

A second, apparently minor BER pathway has been defined in some cells. This pathway has been demonstrated in E. coli, Xenopus laevis, and humans using in vitro systems (9, 14–16). In this pathway, the 5'-deoxyribose phosphate moieties are moved as part of a short oligonucleotide by a structure-specific endonuclease, following strand displacement synthesis at the site of incision by an AP endonuclease. This process generates a repair patch comprising more than 1 nucleotide. In E. coli this structure-specific endonuclease activity may be provided by DNA polymerase I (14, 17), and in mammals by DNase IV (FEN-1) (18). The latter is a member of the yeast Rad2 nucleosome family (19). Its counterpart in yeast is designated Rad27 (20) or Rth1 protein (21).

The major AP endonuclease in Saccharomyces cerevisiae is the APN1 gene product, a homolog of E. coli endonuclease IV (22). Surprisingly, in contrast to the situation in mammals, an exonuclease III homolog is apparently not present in yeast. A structural homolog of the mammalian POLβ gene (which encodes DNA polymerase β) has been identified in yeast (23–25). However, this gene is rarely expressed in vegetative cells (25), and cells deleted of the POLβ gene do not show a defect in DNA repair (23, 24). Instead, DNA polymerase ε has been shown to be required for repair synthesis during BER in yeast (26). At the present time it is unclear as to whether BER in S. cerevisiae is similarly mediated by two pathways and, if so, what the repair patch size is in each. Using a cell-free system, we have examined the incision mechanism and repair patch size during yeast BER. In this report we demonstrate a requirement for the Apn1 protein in BER. Additionally, we have identified a rate-limiting step during BER in this lower eukaryote and we have measured repair patch sizes.

**MATERIALS AND METHODS**

**Strains**—The S. cerevisiae wild-type strains used were CL1265–7C (27) and BJ2168 (28). The apn1 deletion mutant was DRY370 (29).

**Preparation of DNA Substrates for in Vitro Repair**—To prepare osmium tetroxide-damaged DNA, plasmid pUC18 (100 μg) was treated with the agent (300 μg/ml) in 300 μl of TE5 buffer (10 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 100 mM NaCl) at 70 °C for 90 min. Damaged DNA was then purified by centrifugation in a linear 5–20% sucrose gradient.

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§ The abbreviations used are: BER, base excision repair; AP, apurinic/apyrimidinic; dRpase, deoxyribophosphodiesterase.
Single-stranded oligonucleotides (30-mer) containing a uracil or a thymine residue at position 13 were synthesized in a DNA synthesizer. The oligonucleotide was then annealed to its complementary strand. The annealing was carried out by mixing equimolar amounts of both oligonucleotides and incubating for 5 min at 85 °C in TES buffer, followed by slow cooling slowly to room temperature. For incision assays, the uracil-containing strand (top strand) was labeled at the 5' end by [$\gamma$-32P]ATP with T4 polynucleotide kinase prior to annealing. Two sets of 30-mer duplex substrates were used in this study: (a) substrate U and its thymine-containing control substrate T (26), and (b) substrate U-mse1 and its thymine-containing control substrate T-mse1. The nucleotide sequences for these substrates are listed below. For simplicity, only the top strand of each duplex is shown.

**Substrate U:** 5’-GGATGCGATCAUTACGGGAGGCCGCGC-3’

**Substrate T:** 5’-GGATGCGATCATGACGGGAGGCCGCGC-3’

**Substrate U-mse1:** 5’-GGATGCGATCAUTAACCGGAGGCCGCGC-3’

**Substrate T-mse1:** 5’-GGATGCGATCATTACCGGAGGCCGCGC-3’

### Base Excision Repair in Yeast Nuclear Extracts

**In vitro BER** was performed in yeast nuclear extracts. The extracts were prepared as described previously by Wang et al. (28, 30). Standard repair assays were performed as described previously (28, 30) with slight modifications. Standard reaction mixture (50 μl) contained 2 pmol of the 30-mer duplex DNA, 45 mM Hepes-KOH (pH 7.8), 7.4 mM MgCl$_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 20 μM ATP, 2 μM each dATP, dGTP, and dCTP, 8 μM dTTP, 1 μM of [α-$\gamma$-32P]dATP (3,000 Ci/mmoll), 40 mM phosphocreatine (disodium salt), 2.5 μg of creatine phosphokinase (4% w/v). DNA was extracted by phenol/chloroform and precipitated in ethanol. To enhance 30-mer duplex DNA recovery, 120 ng of a 30-mer oligonucleotide were added to the mixture after the repair reaction. Repair products were separated by electrophoresis on a 15% nondenaturing polyacrylamide gel or a 20% denaturing polyacrylamide gel as indicated. Repair syntheses were visualized by autoradiography of the dried gel (nondenaturing gel) or the wet gel (denaturing gel). Repair activity was quantitated by slicing the DNA bands out of the gel for liquid scintillation counting or by estimating the band intensity on the autoradiogram for relative comparisons.

**Incision Assays in Yeast Nuclear Extracts**—Standard in vitro incision assays were performed in 50 μl of reaction mixture containing 30-mer duplex DNA (0.5 pmol) with its uracil-containing strand labeled by [$\gamma$-32P]ATP at the 5’ end, 45 mM Hepes-KOH (pH 7.8), 4.7 mM MgCl$_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 40 μM phosphocreatine (disodium salt), 4% glycerol, 100 μM bovine serum albumin, and 50–80 μg of yeast nuclear extracts. Repair reactions were performed at 33 °C for 2 h and were stopped by adding SDS and proteinase K to 0.5% and 200 μg/ml, respectively. After incubation at 37 °C for 30 min, DNA was extracted by phenol/chloroform and precipitated in ethanol. To enhance 30-mer duplex DNA recovery, 120 ng of a 30-mer oligonucleotide were added to the mixture after the repair reaction. Repair products were separated by electrophoresis on a 15% nondenaturing polyacrylamide gel or a 20% denaturing polyacrylamide gel as indicated. Repair syntheses were visualized by autoradiography of the dried gel (nondenaturing gel) or the wet gel (denaturing gel). Repair activity was quantitated by slicing the DNA bands out of the gel for liquid scintillation counting or by estimating the band intensity on the autoradiogram for relative comparisons.

**dTTP Hydrolysis**—In vitro BER reaction components were mixed with 80 μg of yeast nuclear extracts with or without ATP and the ATP-regenerating creatine phosphokinase as indicated. DNA was excluded from this reaction to avoid the influence of repair synthesis on the concentration of dTTP. The reaction mixture (50 μl) contained 8 μM dTTP and 1 μCi of [α-$\gamma$-32P]dTTP (3,000 Ci/mmoll) and was incubated at 30 °C for 5–120 min. The reaction was stopped by adding 50 μl of 40 mM EDTA. An aliquot of 1 μl was then spotted onto a thin layer chromatography plate (CEL 300 PEO, Macherey-Nagel). After ascending development in a solvent prepared by mixing equimolar amounts of 2M formic acid and 1 M LiCl at 1:1 ratio, the plate was dried. Standard dATP, dGTP, and dTTP mixture (3 μl) containing 10 mM each was loaded onto the same spot prior to the same sample application. dATP, dGTP, and dTTP were identified on the plate by a hand-held UV light, sliced out of the plate, and their radioactivity measured by scintillation counting after adding 5 ml of scintillation fluid.

**3’′ Incision of AP Site**—Before annealing to its complementary strand, the 30-mer uracil-containing oligonucleotide was labeled at the 5′ end with T4 polynucleotide kinase and [$\gamma$-32P]ATP (3,000 Ci/mmoll). The labeled duplex DNA (10 ng) was treated with 2 units of uracil-DNA glycosylase (Life Technologies, Inc.) at 37 °C for 30 min in a buffer (10 μl) containing 70 mM Hepes-KOH (pH 8.0), 1 mM EDTA, and 1 mM DTT. The duplex DNA with an AP site at position 13 was then cleaved at the 3′ end of the AP site by adding Micrococcus luteus pyrimidine dimerase

**FIG. 1. Repair of Uracil-containing DNA in yeast extracts in vitro**. A, DNA repair synthesis. A 30-mer duplex DNA containing a site-specific uracil residue at position 13 (substrate U-mse1, 40 ng) was incubated in yeast nuclear extract (80 μg) of the wild-type strain BJ2168 under standard in vitro BER conditions at 23 °C for 2 h (lane 1). In a separate reaction, purified uracil-DNA glycosylase inhibitor protein (Ugi, 10 units) of bacteriophage PBS2 was also added (lane 2). Repair products were separated by electrophoresis on a 16% denaturing polyacrylamide gel, B, DNA incision. The uracil-containing strand of the 30-mer duplex DNA was labeled with [32P] for T4 polynucleotide kinase and [$\gamma$-32P]ATP prior to annealing as described under “Materials and Methods.” DNA incision assay was performed by incubating 5 ng of the labeled DNA in the nuclear extract of yeast BJ2168 (50 μg) under standard incubation conditions at 23 °C for 2 h without (lane 1) or with the uracil-DNA glycosylase inhibitor protein (lane 2). After incubation, an aliquot of 5 μl was used directly for analysis on a 20% denaturing polyacrylamide gel without DNA purification. Reaction products were visualized by autoradiography. The positions of DNA markers in nucleotides are indicated on the right.

**RESULTS**

**Repair of Uracil-containing DNA in Yeast Cell-free Extracts**—In an effort to understand the molecular mechanism of BER in yeast, we have attempted to dissect the repair pathway in a cell-free system, using a 30-mer duplex oligonucleotide containing a site-specific uracil residue as the repair substrate (see “Materials and Methods”). BER in this cell-free system reflects the complete repair pathway, beginning with damage recognition and ending with DNA ligation (30). BER can be monitored by DNA repair synthesis, which measures the incorporation of radiolabeled nucleotides into repair patches (26, 28, 30). In wild-type yeast extracts, repair synthesis in the uracil-containing oligonucleotide duplex was readily detected (26) (Fig. 1A, lane 1). To determine whether this repair synthesis exclusively reflects BER at the uracil residue, we added uracil-DNA glycosylase inhibitor (Ugi protein) to the reactions. This protein is highly specific for uracil-DNA glycosylase (31) and is expected to effectively inactivate BER initiated by uracil-DNA glycosylase. As shown in Fig. 1A (lane 2), the inhibitor protein completely abolished repair synthesis in the uracil-containing oligonucleotide duplex. The Ugi inhibitor also completely blocked DNA strand incision at the 12th phosphodiester bond of the oligonucleotide by 5′-AP endonuclease (Fig. 1B, lane 2), indicating that the incision is preceded by removal of the uracil residue at position 13 (U13). These results demonstrate that the repair of the uracil-containing oligonucleotide in yeast extracts exclusively reflects BER initiated at the U13 residue by uracil-DNA glycosylase. We estimated that, under the standard assay conditions, approximately 30–40% of the uracil-
In Vitro BER in Yeast Extracts Requires ATP to Maintain Proper dNTP Pools for Repair Synthesis—In contrast to BER reconstituted with purified E. coli proteins (14), repair synthesis during BER in yeast cell-free extracts requires an ATP-regenerating system (Ref. 28 and data not shown). In this study, we have demonstrated that this requirement is to maintain appropriate concentrations of dNTPs. To do this we measured the concentration of dTTP in yeast extracts under standard BER conditions. DNA was excluded from the reaction mixtures to avoid the influence of DNA repair synthesis on the concentration of dTTP. dTTP was added to a final concentration of 8 μM. In the absence of ATP and an ATP-regenerating system, dTTP was quickly hydrolyzed to dTDP and dTMP (Fig. 2). By 30 min only ~10% of the input dTTP remained (~0.8 μM) (Fig. 2). However, in the presence of ATP and an ATP-regenerating system, the dTTP concentration remained constant throughout the entire incubation period, with ~93% of the input dTTP (~7.4 μM) remaining after 2 h of incubation (Fig. 2).

Incision at AP Sites—As indicated earlier, during BER removal of uracil residues is followed by endonucleolytic incision at the resulting AP sites. To examine the incision step, we 5’-end labeled the uracil-containing strand of the oligonucleotide duplex with 32P. The labeled duplex DNA was incubated in yeast nuclear extracts in repair buffer without ATP and dNTPs. Under these conditions incision by an AP endonuclease at AP sites is expected to result in DNA strand breaks, which can be detected by denaturing polyacrylamide gel electrophoresis. Incubation of the labeled DNA with yeast nuclear extracts resulted in incision at the U13 position, releasing labeled 12-mer oligonucleotide fragments exclusively (Fig. 1B, lane 1). Hence, incision takes place 5’ to AP sites, consistent with results reported for the major AP endonuclease activity in many organisms, including yeast (32, 33). The incision reaction is very rapid. By 10 min the majority (>80%) of the input substrate was cleaved at the uracil position (Fig. 3, lane 1), and by 1.5 h essentially all the substrate DNA was cleaved (Fig. 3, lane 4). These results demonstrate that the first two steps of the yeast in vitro BER process, excision of uracil residues from DNA and incision at AP sites, occur rapidly, approaching completion in 10 min.

Requirement for Apn1 Protein for Incisions at AP Sites—To define the protein responsible for catalyzing the AP incision reaction in yeast nuclear extracts, we performed assays in an apn1 deletion mutant extract. As shown in Fig. 4A (lanes 1–5), incision in the uracil-containing oligonucleotide duplex, as evidenced by the formation of a 12-mer DNA fragment, was defective in the apn1 mutant extract, even after 2 h of incubation (Fig. 4A, lane 5). Instead, a small fraction of the AP-containing DNA was cleaved at a different position, leading to the release of DNA fragments, which migrated slightly faster than the 13-mer DNA marker (Fig. 4A, lanes 1–5). This DNA fragment migrated identically to a control substrate sequentially treated with purified uracil-DNA glycosylase and M. luteus pyrimidine dimer-DNA glycosylase, a known 3’-AP lyase (32) (Fig. 4A, lane 11). Thus, this slower incision is apparently effected 3’ to AP sites. Strand cleavage by this 3’-AP lyase mechanism increased slightly with increasing time of incubation, but involved only ~15% of the input DNA after a 2-h incubation (Fig. 4A, lanes 1–5). The inclusion of ATP in the assays did not affect defective 3’-incision or the minor 3’-DNA lyase activity in the apn1 mutant extract (Fig. 4A, lanes 6–10).

Incision 3’ to AP sites by AP lyases is mediated by a β-elimination mechanism, yielding γ-phosphate and a 3’-α,β-unsaturated aldehyde terminus (32). The latter moiety needs to be further processed before repair synthesis can occur. To determine whether the 3’-AP incision in the apn1 mutant extract can be further processed for subsequent DNA repair synthesis, we examined repair synthesis in the uracil-containing oligonucleotide duplex in the apn1 mutant extract. As shown in Fig. 4B, deleting the APN1 gene abolished repair synthesis in the mutant extract.
To directly determine whether BER initiated by a DNA glycosylase with associated AP lyase activity requires a 5'-endonuclease such as the Apn1 protein, we performed DNA repair synthesis in the apn1 mutant extract using osmium tetroxide-damaged pUC18 DNA as the BER substrate. As shown in Fig. 5, repair synthesis was deficient in the apn1 mutant extract. This result could not be explained by the different genetic backgrounds other than the apn1 mutation, since repair with many wild-type strains did not indicate a significant effect of various genetic backgrounds on in vitro repair in yeast extracts (data not shown). To directly show the specific effect of apn1 mutation, we supplemented the mutant extract with purified E. coli endonuclease IV, a 5'-AP endonuclease, and a homolog of the yeast Apn1 protein (22). As shown in Fig. 5 (lane 4), this endonuclease complemented the apn1 mutant extract to proficient repair, indicating that the deficient repair is a result of lacking the Apn1 endonuclease activity in the mutant extract. Deleting the APN1 gene, however, did not completely abolish the repair of osmium tetroxide-damaged DNA, as evidenced by the small amounts of residual repair synthesis in the mutant extract (Fig. 5, lane 3). Thus, efficient processing of 3'-terminus following combined DNA glycosylase and AP lyase activities requires Apn1 protein in yeast extracts.

Collectively, these results show that BER in yeast extracts requires Apn1 protein to effect incision at an AP site. The results suggest that Apn1 also plays a major role in processing the 3'-termini following DNA strand cleavage 3' to the AP sites by an AP lyase or a β-elimination mechanism. Yeast extracts do not appear to contain a redundant 5'-AP endonuclease that can compensate for Apn1 function in its absence.

**Processing of 5'-Deoxyribosyl Phosphate Moieties Is a Rate-limiting Step in BER in Yeast—Incision by Apn1 endonuclease at AP sites leaves 3'-OH and 5'-deoxyribosyl phosphate termini. The latter must be removed during BER. To examine this step, we interrupted the repair of 5'-end labeled uracil-containing oligonucleotide duplexes at the stage immediately following Apn1 incision by incubating in yeast extract for 1 h without ATP and dNTPs (Fig. 6, lane 1). ATP and dNTPs were then added to the reaction mixture to allow completion of repair. In contrast to the fast kinetics of uracil removal and Apn1 incision 5' to AP sites (Fig. 3), repair following Apn1-mediated incision is a slow process, as evidenced by the slow conversion of the incised DNA (12-mer fragment) to the fully repaired product (30-mer fragment) (Fig. 6). Some repair of the incised DNA was detected after 30 min of incubation in the yeast extracts (Fig. 6, lane 2). But even after 2 h of incubation, <40% of the incised DNA was repaired (Fig. 6, lane 5, compare 12-mer and 30-mer DNA bands). In addition, both 13-mer and 17-mer repair intermediates were evident. These represent 1-nucleotide and 5-nucleotide repair synthesis patches, respectively (Fig. 6, lanes 3 and 4). ATP and dNTPs (Fig. 6, lanes 1-5) were added to the reaction mixture to allow completion of repair. Inactivation of the BER substrate. As shown in Fig. 6, some repair of the incised DNA was detected after 30 min of incubation in the yeast extracts (Fig. 6, lane 2). But even after 2 h of incubation, <40% of the incised DNA was repaired (Fig. 6, lane 5, compare 12-mer and 30-mer DNA bands). In addition, both 13-mer and 17-mer repair intermediates were evident. These represent 1-nucleotide and 5-nucleotide repair synthesis patches, respectively (Fig. 6, lanes 3 and 4). ATP and dNTPs (Fig. 6, lanes 1-5) were added to the reaction mixture to allow completion of repair.
FIG. 6. Repair after Apn1 incision. The 32P-labeled uracil-containing DNA was first incised in 50 μg of yeast nuclear extract (wild-type BJ2168) under standard incision conditions at 23 °C for 1 h. ATP, creatine phosphokinase, dATP, dGTP, dCTP, and dTTP (8 μM each) were then added according to the repair synthesis conditions to complete the BER pathway. Post-incision repair was at 23 °C for 0 min (lane 1), 30 min (lane 2), 60 min (lane 3), 90 min (lane 4), and 120 min (lane 5). After repair, 5 μl of the reaction products were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel and DNA bands visualized by autoradiography.

FIG. 7. Repair after removal of the 5′-deoxyribose phosphate moiety in BER pathway. The 32P-labeled uracil-containing DNA was sequentially treated with E. coli uracil-DNA glycosylase and M. luteus pyrimidine dimer-DNA glycosylase (lane 1). Purified E. coli endonuclease IV (~10 ng) was then added and incubation continued at 37 °C for 30 min. Finally, yeast apn1 mutant extract (80 μg) and other repair synthesis components were added to complete the BER pathway. Repair in yeast nuclear extract was at 23 °C for 5 min (lane 2), 60 min (lane 3), and 120 min (lane 4). After repair, 5 μl of the reaction products were analyzed by electrophoresis on a 20% denaturing polyacrylamide gel and DNA bands visualized by autoradiography.

result in an oligonucleotide product containing a single nucleotide gap at position 13, thus effectively creating a repair intermediate in which the processing of 5′-deoxyribose phosphate moieties was bypassed. When we examined repair of this intermediate in the apn1 mutant extract, significant repair synthesis was detected after only 5 min of incubation, as indicated by single nucleotide addition to the cleaved DNA strands that migrated as a 13-mer band (Fig. 7, lane 2). After 1 h of incubation, >90% of the input DNA substrate was repaired and ligated, resulting in the formation of 30-mer DNA strands as completed repair products (Fig. 7, lane 3). Furthermore, the 17-mer repair intermediate was not detected in this experiment (Fig. 7), suggesting that its formation is due to repair synthesis that takes place only in the presence of 5′-deoxyribose phosphate moieties. Since the 13-mer repair intermediate was detected (Fig. 7, lanes 2 and 3), it appears that DNA repair synthesis is faster than DNA ligation. These results show that the DNA repair synthesis and DNA ligation reactions are relatively fast steps during BER in yeast. Hence, we conclude that the slow repair kinetics shown in Fig. 6 is the result of the slow processing of 5′-deoxyribose phosphate moieties and that this is a rate-limiting step during BER.

Repair Patch Size—We performed DNA repair synthesis using the oligonucleotide duplex containing a uracil residue at position 13. Initially repair synthesis was carried out in the presence of only one deoxyribose triphosphate: dTTP (Fig. 8, lanes 1 and 2), dGTP (Fig. 8, lanes 3 and 4), dATP (Fig. 8, lanes 5 and 6), or dCTP (Fig. 8, lanes 7 and 8). In each case, repair synthesis was detected by the corresponding [α-32P]-labeled deoxyribose triphosphate, and compared between the uracil-containing substrate (Fig. 8, lanes 1, 3, and 5) and the control substrate without uracil (Fig. 8, lanes 2, 4, 6, and 8). Only dTTP supported significant "damage" (uracil-specific) repair synthesis (Fig. 8), indicating that repair synthesis begins at the excised uracil residue. Thus, the 3′-terminus formed after Apn1-catalyzed incision is not subject to degradation prior to repair synthesis.

To precisely map repair patches, we performed standard repair synthesis assays in the presence of all four deoxyribose triphosphates and [α-32P]dTTP (Fig. 9A, lane 1). After the reactions the oligonucleotide duplexes were purified and digested with various restriction enzymes. SphI cleavage generated 5′ 10-mer and 3′ 20-mer fragments (Fig. 9A). Digestion of repaired DNA by this enzyme resulted in 32P-labeled 20-mer DNA fragments (Fig. 9B, lane 3), indicating that repair patches were located in 3′ 20-mer SphI fragments. HpaII cleavage generated 5′ 17-mer and 3′ 13-mer fragments (Fig. 9A). Digestion of repaired DNA by this enzyme resulted in 32P-labeled 17-mer DNA fragments (Fig. 9B, lane 4), indicating that 5′ 17-mer HpaII fragments contained repair patches. The small amounts of 32P-labeled 13-mer after HpaII digestion most likely represent low levels of unligated repair products, since this faint band was also observed as a repair intermediate prior to the restriction digestion (Fig. 9B, lanes 1 and 4). To determine if repair synthesis was extended beyond a single nucleotide at position 13, repair products were digested with MseI, which cleaves between residues T13 and T14, generating 5′
13-mer and 3’ 17-mer fragments (Fig. 9A). Both DNA fragments contained 32P-labeled repair patches (Fig. 9B, lane 2). However, the radioactivity in the 3’ 17-mers was ~50% that in the 5’ 13-mers (Fig. 9B, lane 2). Hence, under standard in vitro repair conditions approximately one half of the repaired products contain single nucleotide repair patches, while the rest contain repair patches larger than 1 nucleotide.

To determine the maximal size of repair patches, we labeled them with each of the four [α-32P]deoxyribose triphosphates. Each experiment was performed under identical conditions with the same specific activity of 32P: [α-32P]dTTP (Fig. 10, lanes 1 and 2), [α-32P]dGTP (Fig. 10, lanes 3 and 4), [α-32P]dATP (Fig. 10, lanes 5 and 6), or [α-32P]dCTP (Fig. 10, lanes 7 and 8). In addition to the uracil-containing substrate (Fig. 10, lanes 1, 3, 5, and 7), the oligonucleotide duplex without uracil was used as a control for nonspecific DNA synthesis (Fig. 10, lanes 2, 4, 6, and 8). In the BER repair patches, the first nucleotide (T13) can only be labeled with dTTP, the second nucleotide (G14) with dGTP, the third nucleotide (A15) with dATP, and the fifth nucleotide (C17) with dCTP. Consistent with the earlier experiments (Fig. 9B), the first nucleotide in the repair patches was strongly labeled by dTTP (Fig. 10, lane 1), while the second nucleotide was labeled at ~50% by dGTP (Fig. 10, lane 3). This result indicates that about half of the repair synthesis events were completed after incorporating only 1 nucleotide. Repair synthesis extending to the third nucleotide was even less frequent, as evidenced by even lower levels of incorporation of dATP in repair patches (~25% of that in the first nucleotide position) (Fig. 10, compare lanes 1 and 5). The likelihood of extending the repair patch to the fifth nucleotide was further reduced to <10% of the first nucleotide (Fig. 10, compare lanes 1 and 7). Accordingly, the distribution of the various repair patch sizes in our yeast cell-free system was estimated to be ~50%, 25%, 13%, and 5% for 1-, 2-, 3-, and 5-nucleotide patches, respectively.

**DISCUSSION**

BER of damaged or inappropriate bases is initiated by specific DNA glycosylases (3). Hydrolysis of C–N glycosylc bonds results in AP sites in DNA that are further processed. Using a yeast cell-free system (28, 30), we have examined the incision mechanism and repair patch size during BER of uracil-containing DNA. Under the conditions employed in this study, repair of a uracil-containing 30-mer oligonucleotide duplex is exclusively mediated by BER. This conclusion is based on two observations: 1) repair synthesis was detected in uracil-containing DNA but not in control DNA without uracil residues, and 2) the Ugi (uracil-DNA glycosylase inhibitor) protein of bacteriophage PBS2 completely blocked the repair of uracil-containing DNA.

It has been noted by us and others that BER in cell-free extracts as monitored by DNA repair synthesis requires ATP and an ATP-regenerating system (28, 34). Since reconstituted BER with purified *E. coli* proteins does not show a similar requirement, it is suspected that the role of ATP during BER in cell-free extracts may be merely to maintain appropriate concentrations of input dNTPs for repair synthesis. We have confirmed this explanation by directly measuring dNTP concentrations in yeast extracts with or without ATP. Thus, it is likely that, as is the case in *E. coli*, eukaryotic BER does not have a specific mechanistic requirement for ATP in vivo.

Following the removal of uracil, incision at AP sites in yeast extracts occurs exclusively 5’ to these lesions and is solely dependent on the activity of Apn1 protein. Consistent with these results, *apn1* deletion mutant extracts do not contain detectable 5’-AP endonuclease activity. Hence, Apn1 is the major AP endonuclease in yeast, as suggested previously (33). We have searched the yeast genome, but did not find an *E. coli* exonuclease III homolog. These results indicate that the yeast *S. cerevisiae* contains only one major 5’-AP endonuclease: Apn1. This is in contrast to the situation in *E. coli*, in which at least two major 5’-AP endonuclease activities exist: exonuclease III and endonuclease IV (3). The yeast extracts in our studies were prepared from cells grown under normal condi-

**Fig. 9. Repair patch size analysis.** A, uracil-containing DNA used for repair patch size analysis with indicated restriction sites and resulting DNA fragment sizes. Only the uracil-containing strand of the duplex is shown. B, restriction endonuclease analysis of the repaired products. BER of the uracil-containing DNA (2 pmol) was performed in 80 µg of yeast nuclear extract (BJ2168) at 23 °C for 2 h in the presence of [α-32P]dTPP. After purification, the DNA was digested with *MboI* (lane 2), *SphI* (lane 3), or *HpaII* (lane 4). Repair products without restriction digestion are shown in lane 1. DNA bands were separated by electrophoresis on a 20% denaturing polyacrylamide gel and autoradiographed. DNA size markers in nucleotides are indicated on the left.

**Fig. 10. Labeling repair synthesis with various [α-32P]dNTPs during BER of uracil-containing DNA.** Repair synthesis assays were performed in 80 µg of yeast nuclear extract (BJ2168) in the presence of 8 µM dNTPs and one of the following [α-32P]dNTPs: [α-32P]dTTP (lanes 1 and 2), [α-32P]dGTP (lanes 3 and 4), [α-32P]dATP (lanes 5 and 6), and [α-32P]dCTP (lanes 7 and 8). Repair of the uracil-containing 30-mer duplex DNA (substrate U) is shown in lanes 1, 3, 5, and 7; repair of the thymine-containing DNA (substrate T) is shown in lanes 2, 4, 6, and 8. Repair products were separated by electrophoresis on a 16% denaturing polyacrylamide gel and autoradiographed.
tions. Thus, our results do not exclude the possibility that a putative minor AP endonuclease may play a role during inducible BER. A minor phosphodiesterase/AP site cleavage activity was reported recently in the apn1 mutant (35). However, this activity was not inducible by DNA damage (35). Whether this minor activity plays a role in BER remains to be determined.

Repair of osmium tetroxide-treated DNA was greatly reduced in apn1 mutant extracts. However, unlike the repair of uracil-containing DNA, a low level of residual repair was detected in osmium tetroxide-treated DNA after incubation in apn1 mutant extracts (Fig. 5). This could result from a DNA glycosylase with associated AP lyase, such as a yeast homolog of the E. coli endonuclease III. Indeed, genes encoding the endonuclease III homolog have been isolated recently from the yeast and humans (36–39). The AP lyase may promote β-elimination, eventually leading to the removal of some AP sites in the absence of Apn1. Thus, the processing of AP sites during BER of different base damage may differ slightly depending on whether the process is initiated by a glycosylase with or without associated AP lyase activity. In the former case the AP lyase may contribute to initial incisions at AP sites. Incision 3’ to AP sites during the repair of uracil-containing DNA in apn1 mutant extracts is inefficient and slow (Fig. 4A). However, such incision by a glycosylase with associated AP lyase may be sufficient in the immediate proton of the AP site to the enzyme. Indeed, it has been shown that the glycosylase and lyase activities of E. coli endonuclease III on DNA-containing thymine glycans are inseparable (40). The removal of the damaged base by endonuclease III is immediately followed by cleavage of the resulting AP site (40). This notion argues for a physiological role of AP lyase activity associated with DNA glycosylases. As we have observed during the repair of osmium tetroxide-damaged DNA, such a glycosylase/AP lyase can lead to some repair in the absence of the major 5’-AP endonuclease. However, repair by a glycosylase without associated AP lyase, as in the case of uracil-containing DNA, is totally defective without a 5’-AP endonuclease.

By examining the reaction kinetics of individual steps during the repair of uracil-containing DNA, we found that the processing of 5'-deoxyribose phosphate moieties constitutes a rate-limiting step of BER in yeast. Fast cleavage at AP sites and slow processing of the 5’-deoxyribose phosphate moieties could result in the accumulation of DNA single-strand breaks as significant repair intermediates. DNA strand break intermediate during BER may be temporarily protected by DNA binding proteins in cells.

In yeast cell-free extracts, DNA repair synthesis during BER of uracil-containing DNA incorporated 1 or a few nucleotides. We estimate that ~50% of the repair synthesis events involve only 1 nucleotide in the repair patch, while patches of 2, 3, and 5 nucleotides account for ~25%, 13%, and 5% of the repair events, respectively. This distribution of repair patch sizes varied slightly among extracts prepared from different yeast strains (data not shown). We speculate that this variation may be a result of differences in the efficiency of processing 5’-deoxyribose phosphate moieties by the extracts. We consistently observed small amounts of repair synthesis intermediates representing a few (up to 5) nucleotides incorporated without DNA ligation. These intermediates were not observed if repair synthesis was performed after removing 5’-deoxyribose phosphate moieties by an AP lyase- and endonuclease IV-mediated cleavages at AP sites. Thus, it is likely that repair patches longer than 1 nucleotide result from repair synthesis in the presence of unprocessed 5’-deoxyribose phosphate moieties. Similar conclusions were reached based on in vitro studies of BER in E. coli (14). In such a mechanism, a short stretch of nucleotides containing the 5’-deoxyribose phosphate moiety would have to be cleaved prior to DNA ligation. This cleavage can be effected by the nuclease activity of DNA polymerase I in E. coli (14). In yeast, a candidate for such an activity is the Rad27 protein (19). In support of this hypothesis, rad27 deletion mutants are sensitive to several DNA-damaging agents (20).

Our results with regard to repair patch sizes during BER in yeast extracts are in agreement with those of E. coli and human systems (18, 14, 15). Apparently the basic mechanism of BER has been well conserved, underscoring the physiological importance of this excision repair mode in the cellular response to many DNA-damaging agents. The formation of repair patches of 1 or a few nucleotides during BER has been attributed to two repair pathways (9, 14, 15). In higher eukaryotes, the major pathway appears to be a DNA polymerase β-dependent one, incorporating a single nucleotide into the patch (9, 11, 12). A second minor pathway is proliferating cell nuclear antigen-dependent, incorporating a few (up to 7) nucleotides. The latter pathway requires DNA polymerase δ and/or ε (9, 15, 16). In yeast, DNA polymerase β is rarely expressed in vegetative cells (25). Consistently, its deletion does not affect cellular sensitivity to DNA-damaging agents (23, 24). In contrast, DNA polymerase ε has been found to be a major enzyme operating during BER in yeast (26). This is reminiscent of E. coli BER, where DNA polymerase I can lead to a repair patch of 1 or a few nucleotides (14).

We consider the processing of 5’-deoxyribose phosphate moieties to be the primary mechanism that determines the operation of the two BER pathways. When 5’-deoxyribose phosphate moieties are removed by a dRpase, a single-nucleotide gap is generated. However, this is a relatively slow reaction. Hence, an alternative mechanism is employed to enhance the efficiency of this rate-limiting step, by incorporating a few nucleotides into repair patches during DNA strand displacement synthesis. This facilitates the removal of terminal 5’-deoxyribose phosphate moieties by a structure-specific endonuclease cleaving at the single-stranded and the double-stranded junction. This mechanism provides a satisfactory explanation for the observation that the major BER polymerase (polβ) in higher eukaryotes has associated dRpase activity (8). Employment of DNA polymerase β for repair synthesis is expected to increase the overall repair efficiency during BER, because the rate-limiting reaction is enhanced by the associated dRpase activity. The existence of two BER pathways provides even further repair efficiency as well as functional redundancy. Hence, defective or inefficient processing of the rate-limiting reaction in BER by the major repair pathway could be partially substituted by the second repair pathway.

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