hUNG2 Is the Major Repair Enzyme for Removal of Uracil from U:A Matches, U:G Mismatches, and U in Single-stranded DNA, with hSMUG1 as a Broad Specificity Backup*

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hUNG2 and hSMUG1 are the only known glycosylases that may remove uracil from both double- and single-stranded DNA in nuclear chromatin, but their relative contribution to base excision repair remains elusive. The present study demonstrates that both enzymes are strongly stimulated by physiological concentrations of Mg²⁺, at which the activity of hUNG2 is 2–3 orders of magnitude higher than that of hSMUG1. Moreover, Mg²⁺ increases the preference of hUNG2 toward uracil in ssDNA nearly 40-fold. APE1 has a strong stimulatory effect on hSMUG1 against dsU, apparently because of enhanced dissociation of hSMUG1 from AP sites in dsDNA. hSMUG1 also has a broader substrate specificity than hUNG2, including 5-hydroxymethyluracil and 3,N⁴-ethenocytosine. hUNG2 is excluded from, whereas hSMUG1 accumulates in, nucleoli in living cells. In contrast, only hUNG2 accumulates in replication foci in the S-phase. hUNG2 in nuclear extracts initiates base excision repair of plasmids containing either U:A and U:G in vitro. Moreover, an additional but delayed repair of the U:G plasmid is observed that is not inhibited by neutralizing antibodies against hUNG2 or hSMUG1. We propose a model in which hUNG2 is responsible for both prereplicative removal of deaminated cytosine and postreplicative removal of misincorporated uracil at the replication fork. We also provide evidence that hUNG2 is the major enzyme for removal of deaminated cytosine outside of replication foci, with hSMUG1 acting as a broad specificity backup.

Uracil in DNA can be introduced via two mechanisms, deamination of cytosine and misincorporation of dUMP during replication. Deamination of cytosine has been calculated from measured deamination rates to occur at a rate of 100–500 per human cell/day (1, 2) to yield mutagenic U:G mispairs. Uracil may also appear as a consequence of misincorporation of dUMP instead of dTMP during replication, resulting in a U:A base pair. The latter is not miscoding, but may produce cytotoxic and mutagenic AP site intermediates during repair. In organisms containing 5-methylcytosine in their genomes, deamination of 5-methylcytosine furthermore leads to T:G mismatches. All living organisms express uracil-DNA glycosylases (UDGs)¹ that prevent cytotoxic and mutagenic effects of the above lesions. UDGs remove uracil (and sometimes other damaged bases or thymine) from the deoxyribose and thus initiate a multistep base excision repair (BER) pathway, eventually restoring the correct DNA sequence. After removal of uracil by an UDG and cleavage of the resulting abasic site by AP endonuclease (APE1/APE2), the BER pathway splits into two branches (reviewed in Ref. 3). The presumed major track is the short-patch pathway. It uses the 5’-deoxyribophosphodiesterase activity of DNA polymerase β to cleave 3’ of the abasic site, thus releasing deoxyribose-5-phosphate. Then pol β inserts C or T, depending on the template base. Finally, DNA ligase III seals the nick, perhaps aided by the scaffold protein XRCC1. The alternative long-patch pathway largely uses replication proteins and may take place in replication foci (4). This pathway requires pol ε and/or δ, as well as the trimeric sliding clamp and polymerase processivity factor proliferating cell nuclear antigen (PCNA) and the clamp loader replication factor C (RFC). Repair synthesis is stimulated by pol β, which may be important in the first step of polymerization. The structure-specific endonuclease FEN1 removes the 2–8-nucleotide displaced “flap” of DNA, and DNA ligase I seals the nick (3).

Mammalian cells contain at least four UDGs, of which three (UNG, SMUG1, and TDG) belong to the same protein superfamily, possess the same fold, and have probably evolved from a common ancestor (5). Of these, UNG appears to be quantitatively dominating as determined from activity assays using human cell-free extracts and U:A substrates (6). UNG belongs to the family of highly conserved UDGs typified by Escherichia coli Ung, and is present in a large number of eukaryotes, bacteria, and large eukaryotic DNA viruses (7). The human and mouse UNG genes encode both mitochondrial (UNG1) and nuclear (UNG2) forms of the enzyme by way of alternative promoter usage and mRNA splicing (8). The catalytic domain of hUNG has been extensively studied, and its structure and molecular mechanism of catalysis and specificity established (9–12). The enzyme removes uracil in vitro in the order of

¹ The abbreviations used are: UDG, uracil-DNA glycosylase; BER, base excision repair; APE, AP endonuclease; PCNA, proliferating cell nuclear antigen; pol, polymerase; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; FU, fluorouracil; cC, 3,N⁴-ethenocytosine; ss, single-stranded; ds, double-stranded; NLS, nuclear localization signal; Hmt1, hydroxymethyluracil; DTT, dithiothreitol; EGFP, enhanced green fluorescent protein; BSA, bovine serum albumin; 5mC, 5-methylcytosine.

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preference ssU > U-G > U-A (6). Certain closely related bases formed from cytosine after γ-irradiation or oxidative stress are also substrates, such as 5-hydroxycytosine, isodiolactic acid, and alloxan (13). These are, however, excised at a very low rate compared with uracil. A second human UDG against both ss- and dsU, hSMUG1 (single-strand selective multifunctional uracil-DNA glycosylase), was identified more recently by in vitro expression cloning (14). hSMUG1 is not found in bacteria and yeast, but is present in higher eukaryotes. Although they probably share the same fold and motifs necessary for substrate binding and catalysis as hUNG, little homology exists between the two enzymes at the amino acid level (5). hSMUG1 is furthermore located in the nuclei, and Xenopus SMUG1 has a substrate preference similar to the catalytic hUNG domain (ssU > U-G > U-A), although its specific activity is considerably lower (14). Recently, Boorstein and co-workers (15) demonstrated that hSMUG1 is also active against 5-hydroxymethyluracil (5hmU). 5hmU is formed in DNA by oxidative attack on the methyl group of thymine, thereby creating 5hmU:A. It is also the product of the deamination of 5-hydroxymethylcytosine, which may be formed via oxidation of 5-methylcytosine. The latter creates a HmU-G base pair, which would be mutagenic if left un repaired. Although HmU-G substrates were not tested, the authors suggested the latter could be a biologically important substrate (15). This is also supported by the fact that transition mutation from 5mC-G to T-A is the most frequent substitution mutation in human cancer (16). The two last UDGs identified in human cells, TDG and MBD4 (MED1), are both mismatch-specific and have no activity against single-stranded substrates. TDG excises uracil and thymine from U-G and T:G mismatches, as well as 3,4N²-ethenocytosine (εC) and 5-fluorouracil (5FU) from double-stranded DNA, and may restore G:C base pairs at sites of cytosine or 5-methylcytosine deamination, or alklylation, respectively (17). MBD4, which does not belong to the same superfamily as the three other UDGs, acts on uracil, thymine, 5-FU, and εC mispaired with guanine (18), as well as on 5-methylcytosine at hemimethylated DNA (19). The preferred substrates, however, are G:T mismatches at methylated or unmethylated CpG islands. Thus, MBD4, as well as TDG, may have a function in the correction of T:G mismatches originating from deamination of 5-methylcytosine.

Several lines of evidence indicate that nuclear UNG2 has a major role in postreplicative removal of misincorporated uracil in mammalian cells (4). The contribution of UNG2 to repair of deaminated cytosines has, however, been debated. Whereas bacterial and yeast ung mutants display a mutator phenotype unable to repair deaminated cytosines (20, 21), such a phenotype is not clearly observed in Ung⁻ mice (22). Based on this finding, and comparison of kinetic parameters of the Xenopus SMUG1 (14) and the hUNG catalytic domain (6), it was suggested that in higher eukaryotes, the contribution of UNG2 to the excision of deaminated cytosines was reduced, and that this function might instead be provided by SMUG1 (23). In human cells, however, the UNG catalytic domain alone is not observed in the nucleus (24). Rather, the entire N-terminal regulatory domain remains attached to the core catalytic domain after nuclear translocation. Until now, little information has existed on the enzymatic properties of full-length hUNG2. A likely reason for this is the susceptibility of the enzyme to N-terminal proteolytic degradation during purification, mainly resulting in the core catalytic domain (25). To gain further insight in the functional properties of hUNG2 and hSMUG1 and their relative contribution to nuclear base excision repair, both proteins were purified after overexpression in E. coli. In depth biochemical characterization and analysis of subnuclear localization revealed previously unrecognized properties of both hUNG2 and hSMUG1 that may have important implications for their functions in nuclear BER in vivo.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant hUNG2—An NdeI site was introduced at the hUNG2 translation start codon, and a 947-bp Ndel/HpaI fragment encompassing the entire hUNG2 coding region was ligated into the new vector pJ685Krop251kan. This expression vector was constructed by introducing a high copy number variant of the trpA gene (Arg251→Met) and changing the resistance from ampicillin to kanamycin in the toluic acid-inducible, broad host vector pUBS55 (26). The resultant construct, p685Kung2, was introduced into E. coli BL21. Fermentation, preparation of crude extract, and the initial chromatographic steps were essentially as described for the hUNG catalytic domain (6) except that the culture was induced by 1 mM tolcapone (final) and allowed to grow for additionally 3 h at 30°C before harvesting. Furthermore, Complete™ mini (EDTA-free) (Roche) protease inhibitor tablets were included in the initial steps of the purification (1 tablet/10 ml during homogenization and 1 tablet/ml in subsequent buffers). During size exclusion chromatography on Superdex 75 26/60 (Amersham Biosciences), buffer was changed to 20 mM HEPES-NaOH (pH 8.0), 100 mM NaCl, 1 mM DTT. Pooled active fractions were loaded onto a MonoS HR 5/5 column, and eluted with a linear NaCl gradient from 100 mM to 1 M NaCl in the same buffer. To remove partially N-terminally deleted species, the purest fractions were subjected to MonoS rechromatography and fractions containing the highest portion of apparently full-length protein were pooled. The purified protein was finally concentrated by ultrafiltration and snap-frozen in liquid N2 prior to storage at −80°C.

**Expression and Purification of Recombinant hSMUG—**IMAGE clone identification no. 726197 containing the hSMUG1 cDNA in pT7T3D was cut using Ndel and BglI, and the 1154-bp fragment encoding hSMUG1 was cloned into the Ndel site of PET11a (Invitrogen). The resultant vector was transformed into E. coli BL21-CodonPlus(DE3)-RIL (Stratagene) and bacterial cell mass for purification produced by fermentation. Expression was induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside (final) at 37°C and the culture allowed to grow for additional 4 h prior to cell harvest. Preparation of the hSMUG1 crude extract was essentially as described for the hUNG catalytic domain (6), except that homogenization and protease sulfite precipitation was performed in 20 mM HEPES-NaOH (pH 6.8), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 1 tablet/10 ml Complete® mini (EDTA-free) protease inhibitor mixture (buffer A). The proteamine sulfate fraction was loaded onto a DEAE-Sephacel column (Amersham Biosciences, 5×9 cm) coupled in series with a CM-Sepharose column (Amersham Biosciences, 5×5.5 cm). After washing to base-line absorbance, the DEAE-Sephacel column was bypassed and adsorbed proteins were eluted in a 0.05–1 M NaCl gradient in buffer A. Active fractions were pooled and dialyzed against 20 mM HEPES-NaOH (pH 8.0), 10 mM NaCl, 1 mM DTT (buffer B) and applied to a MonoS-12 column (Bio-Rad). Adsorbed proteins were eluted in a 0.01–0.7 M linear NaCl gradient. Active fractions were pooled and concentrated by ultrafiltration to 5 ml and loaded onto a Superdex 75 HiLoad 26/60 column (Amersham Biosciences) preequilibrated with buffer B containing 100 mM NaCl, and eluted with the same buffer. Fractions containing hSMUG1 were verified by SDS-PAGE and pooled. After a 4-fold dilution in buffer B, hSMUG1 was loaded onto a poly(U)-Sepharose column (Amersham Biosciences, 1.6×10 cm) pre-equilibrated with buffer B containing 2 mM EDTA. Adsorbed proteins were eluted in a 0.01–1 M linear NaCl gradient in the same buffer and the fractions containing hSMUG1 identified by SDS-PAGE and pooled. The poly(U)-Sepharose fraction was then dialyzed against buffer B, applied onto a MonoS HR 5/5 column, and eluted in a linear 0.01–1 M NaCl gradient in the same buffer. The purest hSMUG1 fractions were collected and the remaining hSMUG1-containing fractions rechromatographed on MonoS as above. The pooled hSMUG1 fraction was apparently homogeneous as determined by SDS-PAGE and silver staining. The purified protein was finally concentrated by ultrafiltration and snap-frozen in liquid N2 prior to storage at −80°C.

**Neutralizing Antibodies against hUNG2 and hSMUG1—**Polyclonal PU101 against the hUNG catalytic domain was prepared as described previously (28). Polyclonal PSM1 against the hSMUG1 coding region was produced by immunization (10). The purified protein was finally concentrated by ultrafiltration and snap-frozen in liquid N2 prior to storage at −80°C.
vals, and the final bleed 10 days after the last immunization. IgG-fractions of 1501 and 351 were purified on protein A-Sepharose HiTrap columns (Amersham Biosciences) before being used in inhi-

Cell Culture and Preparation of Nuclear Extracts—Spontaneously transformed human keratinocytes HaCaT, colorectal carcinoma CX-1, and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum, 0.05% glutamine, and 0.1 mg/ml gentamicin at 5% CO2. Cells were harvested in the logarithmic growth phase by trypsinization, washed twice in ice-cold phosphate-buffered saline prior to isolation of nuclei. Subsequently, CX-1 and HeLa were washed once in a hypotonic buffer (isotonic buffer without sucrose) containing Com-

The construct carrying the hUNG2 promoter, pGL2-P A (27), was obtained by restriction enzyme digestion of pUNG2-EYFP. The I fragment from pUNG2-EYFP was then ligated with pEYFP-N1 (Clontech) by transferring the complementary strand containing either A or G opposite U or HmU. The labeled strands were annealed to a 50% excess of the unlabeled strand and heated at 90 °C for 5 min. The mixture was cooled to 37 °C in the presence of T4 DNA polymerase, T4 DNA ligase, and 5 mM MgCl2 or 7.5 mM MgCl2 and 0.44 ng/µl of enzyme. The activity of hUNG2 was measured in the presence of either 7.5 mM MgCl2 or 7.5 mM MgCl2 and 0.44 ng/µl of enzyme. The activity of hUNG2 was calculated as above. In separate experiments, the activities of hUNG2 and hSMUG1 were directly compared by using ds oligonucleotides (20 µM) complementary to the region of the BER plasmid substrates. These were prepared by 5'-end labeling of 19-mer PAGE-purified oligonucleotides containing U or HmU at a concentration of 10 ng/µl. The repair mixtures were incubated at 30 °C for the given times, stopped by addition of EDTA and RNase A, and further analyzed by electrophoresis on 12% denaturing polyacrylamide gels and phosphorimaging of the dried gels.

The base excision repair mixtures (50 µl) contained (final) 40 mM HEPES-KOH (pH 7.8), 70 mM KCl, 5 mM MgCl2, 0.5 mM DTT, 2 mM ATP, 20 µM dATP, 20 µM dGTP, 8 µM dTTP or dCTP depending on the isotope used, 5 µM phosphocreatine, 0.36 mg/ml BSA, 1 µM creatine phosphokinase, 40 µCi/ml [3H]dUMP-containing calf thymus DNA (specific activity of 17 Ci/ml or 6.3 µCi/ml) and varying amounts of enzyme. The mixture was incubated at 30 °C for the given time, stopped by addition of EDTA and RNase A, and further incubated with proteinase K (30 min) and SDS (10 min) at 37 °C. The repair product was recovered by phenol/chloroform extraction and etha-

The mixture was incubated at 30 °C for the given time, stopped by addition of EDTA and RNase A, and further incubated with proteinase K (30 min) and SDS (10 min) at 37 °C. The repair product was recovered by phenol/chloroform extraction and ethanol precipitation. The DNA was digested using XbaI/HinfI for the XbaI/BglII long-patch BER assay and XbaI/HindIII/BglII for the short-patch BER assay. The DNA fragments were then analyzed by electrophoresis in 12% denaturing polyacrylamide gels and phosphorimaging of the dried gels.

To specifically monitor the glycosylase and AP endonuclease steps in the BER-assays, 50-mer double stranded oligonucleotides were prepared that corresponded to the region of the BER plasmid substrates containing glycosylase or AP endonuclease enzymes NheI and AgeI. The products were then digested with T4 DNA polymerase and subsequently cloned into the pECFP-C1 vector. The pECFP-C1 plasmid was digested with NotI to create two fragments, NheI and AgeI, the fragment was cloned into the corresponding sites in the pECFP-C1 vectors (Clontech). The resulting vector was digested with ScaI, the 3' overhang removed by T4 DNA polymerase, and the vector re-ligated to make EYFP-SMUG1. The fusion constructs were verified to be in-frame by sequencing.

The construct carrying the hUNG2 promoter, pGL2-P A (27), was digested with HindIII/EcoRV to remove the luciferase gene. The HindIII/EcoRV fragment from pUNG2-E2YFP was then ligated with HindIII/Eco-

The EcoRI and BglII fragments of pUNG2-EYFP were excised and ligated into the NotI and EcoRV sites of pEYFP-N1 (Clontech) to give pEYFP-N1 (Clontech). The EcoRI/BglII fragment was used to obtain the NheI/AgeI fragment from pEYFP-N1 (Clontech). The resulting fragment was ligated into the pGL2-P A plasmid to give pGL2-P A (27).

The 514-nm laser line for EYFP (detected at 520 nm) and the 541-nm laser line for EYFP (detected at 560 nm).

UDG Activity Assays—Unless otherwise stated, UDG activity was measured in 20 µl of assay mixture containing (final) 20 mM Tris–HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mg/ml bovine serum albumin, 1.8 µM [3H]dUTP-containing calf thymus DNA (specific activity 0.5 µCi/µmol) and varying amounts of enzyme. The mixture was incubated at 10 min at 30 °C, and the amount of released uracil measured as described (29). Kinetic assays using 0.24–21.5 µM calf thymus sub-

The kinetic parameters were measured in the presence of either 7.5 mM MgCl2 or 7.5 mM MgCl2 and 0.44 ng/µl of enzyme. The activity of UDG was calculated as the ratio of APEI/glycosylase at least 10/1). The amount of hUNG2 or hSMUG1 used in the kinetic assays was adjusted to ensure that less than 30% of the substrate was consumed, to ensure linearity of the assay. Kinetic parameters were calculated using the Enzpack for Win-

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BER assay, except that dNTPs were replaced by ddCTP or ddTTP to arrest polymerization and ligation. The amount of AP sites or incised product was quantified as described above.

RESULTS

Purification of Recombinant hUNG2 and hSMUG1—We have found full-length hUNG2 to be notoriously difficult to express and purify from various E. coli strains and budding yeast (Pichia) caused by N-terminal proteolysis. Apparently, this was caused by both in vivo proteolytic attack and degradation during purification (data not shown). The degradation problem was partially overcome by expressing the protein containing an N-terminal His tag. For functional analysis we decided to avoid histidine tags, however, because this may seriously affect the functional properties of some proteins (31).

In the present system, the highest relative amount of full-length hUNG2 at the time of cell harvest was observed using E. coli BL21 (ompT−, lon−), in which −50% of the protein appeared unprocessed. The presence of mixed protease-inhibitors during purification only partially prevented further loss of the intact protein, which generated enzyme species lacking one or a few of the N-terminal residues. These results indicate that the hUNG2 N-terminal regulatory region constitutes a distinct domain sensitive to proteolysis. This would also explain why previous attempts to purify UNG from mammalian cells in the absence of appropriate protease inhibitors have yielded essentially the catalytic domain (25). This is also corroborated by the abnormal chromatographic behavior of hUNG2. The full-length protein (34.6 kDa) eluted as a 52-kDa protein in size-exclusion chromatography, whereas the compact, spherical catalytic domain (25.5 kDa) eluted as a 20-kDa protein (data not shown). Furthermore, hUNG2 eluted over a broad NaCl range in MonoS chromatography, in contrast to the sharp peak observed with the catalytic domain alone. This may indicate that the hUNG2 N-terminal is less ordered than the catalytic domain, and may adopt alternative conformations.

The purified enzyme after repeated MonoS chromatography was apparently homogeneous as judged by SDS chromatography and silver staining. N-terminal sequencing, however, revealed that this fraction contained −60% full-length hUNG2, and −40% of UNG2Δ1 lacking the N-terminal methionine. This was judged adequate for biochemical characterization of the enzyme, and no further attempts were made to remove the 1-amino acid truncated form. Approximately 1 mg of purified, full-length hUNG2 was obtained from a 5-liter fermentation. hSMUG1 was expressed as a full-length protein. However, the hSMUG1 N terminus also proved to be highly susceptible to proteolytic attack even in the presence of mixed protease inhibitors, and virtually all hSMUG1 obtained after purification proved to lack the 16 N-terminal amino acids. The yield was −5 mg of protein/5 liters of fermenter culture. No significant difference in enzymatic activity was observed with the Δ16 form as compared with full-length hSMUG1 expressed using another vector system (pTYB12; data not shown). The former was, however, chosen for biochemical analysis because of the very low yield from the pTYB12 system.

Both hUNG2 and hSMUG1 Are Stimulated by Mg2+ and APE1—To analyze the kinetic properties of hUNG2 and hSMUG1, we first established reaction conditions under which both activities could be directly compared. The pH profile of each enzyme was broad, with an optimum between pH 7.0 and 7.5 (data not shown). Furthermore, hUNG2 was stimulated about 2-fold by 50 mM NaCl (Fig. 1A), whereas hSMUG1 was −30% stimulated at 70 mM NaCl (Fig. 1B). Both the pH profiles and NaCl optima are similar to those reported for the major UDG activity partially purified from HeLa cells (29). In the latter study, the authors also observed a weak stimulation of the activity by Mg2+. To our knowledge, this has not been reported for any other monofunctional DNA glycosylase, including UNG, and it was thus of interest to analyze whether this could be caused by stimulation of co-purifying hSMUG1 in their study. To our surprise, however, both enzymes were markedly stimulated by the presence of Mg2+. In the absence of monovalent cations, hUNG2 was stimulated nearly 10-fold in the presence of 10 mM MgCl2 (Fig. 1A), whereas a near 2-fold stimulation was observed for hSMUG1 (Fig. 1B). This is in contrast to properties of the catalytic core form of hUNG, which is inhibited by Mg2+ at all concentrations tested (data not shown). Thus the N-terminal 86 amino acids in hUNG2 are required for the observed Mg2+-stimulation. Because hSMUG1 and hUNG2 are both nuclear proteins, and the hUNG2 N-terminal region is retained after nuclear translocation (24, 32), we decided to undertake an in-depth study of the kinetic effects of magnesium on both enzymes. An additional advantage of studies of effects of Mg2+ on hUNG2 and hSMUG1 was that their properties could be analyzed in the presence of fully active APE1, which catalyzes the next step in the BER pathway. APE1 has been demonstrated to affect the catalytic rate of several DNA glycosylases (33, 34) and requires Mg2+ for full catalytic activity, but not for DNA binding (35).

The effects of Mg2+ on single-stranded (ssU) DNA substrates in the absence and presence of APE1 are shown in Fig. 1, C (hUNG2) and D (hSMUG1). In the absence of Mg2+, the activities of hUNG2 against ss- and ds-[3H]dUMP-containing calf thymus DNA (U opposite A) were

![Fig. 1. Modulation of hUNG2 and hSMUG1 activities by NaCl, Mg2+, and APE1.](image-url)
respectively. In addition, the stimulatory effect by APE1 mark-
APE1 in the presence of 10 mM Mg2

hUNG2 increased

picture dramatically changed as the ssU/dsU specificity ratio of

increase in

k

Mg2

of Mg 2

presence of APE1 had only a weak stimulatory effect with both

the relative ssU/dsU activity did not change. As for hUNG2, the

Increasing the Mg2

tivity against dsU was observed in the presence of APE1.

stranded regions of DNA. A similar effect was not observed for

substrates.

To further analyze this complex pat-

concentration resulted in a gradual in -

uation experiments (23). It is also less obvious why APE1

for dsU and ssU, whereas

k

Mg2

was quantitatively higher with dsU. Thus, in the presence of

hUNG2 is neither inhibited by AP sites in ssDNA nor dsDNA,

When both Mg2

and APE1 were included in the assays, a

further increase in k_{cat}/K_{m} was observed for hUNG2 against
dsU, whereas k_{cat}/K_{m} against ssU was reduced by 50% com-
pared with Mg2

alone. However, hUNG2 still maintained a

7.4-fold preference for ssU over dsU. For hSMUG1, the opposite
effect was observed in the presence of APE1. The affinity and
turnover against both ssU and dsU increased, but the increase

was quantitatively higher with dsU. Thus, in the presence of

both Mg2

and APE1, hSMUG1 displays a 2-fold preference for the
double-stranded substrate.

AP Sites Inhibit hSMUG1 but Not hUNG2—AP sites have
een shown to be a strong (micromolar range) competitive
inhibitor of the catalytic hUNG domain, and this domain binds
to AP sites more strongly and more rapidly than to uracil-
containing DNA (33). Such binding is also observed with sev-

eral other DNA glycosylases (36, 37). We postulated that bind-
ging to the product AP site could be of crucial importance to
avert mutagenic and cytotoxic effects of the AP site until the
subsequent AP endonuclease arrived and ensured further proc-
dessing of the damage site (33). Surprisingly, no inhibition by
AP sites was observed with the full-length hUNG2 protein
when compared with corresponding non-AP-containing oligo-
nucleotides (Fig. 2A). An entirely different effect was observed
with hSMUG1. Whereas no specific inhibition was observed with
the AP-containing ss-oligonucleotides, a strong inhibitory
effect was observed with the AP-containing ds-oligonucleotides
(Fig. 2B). Furthermore, the AP-G construct inhibited somewhat
more strongly than AP-A. These results may explain the 15-fold
increase in k_{cat}/K_{m} for hSMUG1 against dsU when APE1 is
present (Table I). Because APE1 does not stimulate this activity
in the absence of Mg2

(Fig. 1C), this likely occurs mainly by

Mg2

-stimulated endonucleolytic cleavage of the AP site,
whereby hSMUG1 product rebinding is blocked. hSMUG1 dis-
placement from ds AP sites by APE1, similar to human hTDG
(36) may, however, be a contributing factor, because a weak
stimulation by APE1 is also observed in the absence of Mg2

(Fig. 1D) (note that APE1 binds to AP sites in the absence of

Mg2

, but cleaves at a very reduced rate). Because single-
stranded AP sites do not inhibit hSMUG1, such a mechanism
cannot explain the observed 2-fold increase in k_{cat}/K_{m} of

hSMUG1 against ssU by APE1. Active recruitment of hSMUG1
to the APE1-DNA or formation of a hSMUG1-APE1 complex
prior to substrate binding could hypothetically contribute to
this, but no such interaction has been observed in immuno-
precipitation experiments (23). It is also less obvious why APE1
only stimulates the activity of hUNG2 against dsU. Because
hUNG2 is neither inhibited by AP sites in ssDNA nor dsDNA,
and because the major effect of APE1 is on the K_{m} against both
substrates, it is tempting to speculate that APE1 may directly

essentially identical. Moreover, a weak stimulation of the ac-
tivity against dsU was observed in the presence of APE1.
Increasing the Mg2

concentration resulted in a gradual in-
crease in activity up to 6 and 10 mM Mg2

for dsU and ssU,
respectively. In addition, the stimulatory effect by APE1 mark-
edly increased for dsU in the presence of Mg2

(Fig. 1C). A somewhat different pattern was observed for hSMUG1 (Fig.
1D). In the absence of Mg2

, hSMUG1 had ~4-fold higher activity against
ssU than dsU. The activity against both substrates increased with increasing Mg2

up to 6 mM, although the relative ssUs dsU activity did not change. As for hUNG2, the

presence of APE1 had only a weak stimulatory effect with both
substrates in the absence of Mg2

. However, in the presence of

Mg2

, APE1 had a marked stimulatory effect. This was most
pronounced for dsU, as the activity was stimulated ~10-fold by
APE1 in the presence of 10 mM Mg2

.

In separate experiments, potential stimulation of hUNG2 and
hSMUG1 by the second human AP endonuclease APE2 and
the Flap endonuclease FEN1 was investigated. Neither of
these proteins, however, had any effect on the glycosylase ac-

Table I

Kinetic constants of hUNG2 and hSMUG1 against [3H]dUMP-containing calf thymus DNA and their modulation by MgCl2 and APE1

| Enzyme   | Substrate | MgCl2 (7.5 mM) | APE1 | K_{m} | k_{cat} | k_{cat}/K_{m} |
|----------|-----------|----------------|------|-------|---------|---------------|
| hUNG2    | U:A-ds    | 3.0 ± 0.4      |      | 187 ± 10 | 208     |               |
| hUNG2    | U:A-ds    | 2.9 ± 0.4      |      | 603 ± 29 |          |               |
| hUNG2    | U:A-ds    | 1.2 ± 0.1      |      | 903 ± 29 | 753     |               |
| hUNG2    | U-ss      | 13.8 ± 2.2     |      | 1060 ± 107 | 79      |               |
| hUNG2    | U-ss      | 0.1 ± 0.03     |      | 1005 ± 31 | 10,050  |               |
| hUNG2    | U-ss      | 0.21 ± 0.02    |      | 1178 ± 17 | 5,609   |               |
| hSMUG1   | U:A-ds    | 0.8 ± 0.1      |      | 1.2 ± 0.0 | 1.5     |               |
| hSMUG1   | U:A-ds    | 1.8 ± 0.3      |      | 5.8 ± 0.3 | 3.2     |               |
| hSMUG1   | U:A-ds    | 0.5 ± 0.1      |      | 23.6 ± 0.6 | 47      |               |
| hSMUG1   | U-ss      | 1.7 ± 0.2      |      | 4.0 ± 0.1 | 2.4     |               |
| hSMUG1   | U-ss      | 2.3 ± 0.3      |      | 28.0 ± 1.5 | 12      |               |
| hSMUG1   | U-ss      | 1.8 ± 0.2      |      | 43.6 ± 1.7 | 24      |               |
interact with hUNG2 and regulate its relative affinity against single-stranded versus double-stranded substrates. Preliminary data from our laboratory indicate that direct APE1/UNG2 interactions in fact do occur in vitro, and that this interaction depends on the phosphorylation patterns of hUNG2. This is now under further investigation.

hSMUG1 Has Broader Substrate Specificity than hUNG2—Boorstein and co-workers (15) recently demonstrated that recombinant hSMUG1 and SMUG1 purified from calf thymus were able to excise HMU from DNA (HmU:A) in addition to U, although a direct comparison between the two substrates was not performed. To investigate the relative efficiency of hUNG2 and hSMUG1 against different uracil analogs, various concentrations of both enzymes were incubated with a panel of double-stranded oligonucleotides containing U,G,FU,A,HmU,G,εC,G (Fig. 3), and 5-OHU. The results indicated narrow substrate specificity for hUNG2 restricted to uracil and uracil analogs with minor structural modifications at the 5-position (substrate preference: U >> 5-FU). In contrast, much broader substrate specificity was observed for hSMUG1. In addition to previously reported U and HmU, the enzyme was also active against FU and εC, with the order of preference U > HmU >> εC > FU. Neither enzyme was able to excise 5-OHU under the present conditions (data not shown). These results indicate considerable structural differences in the active site of hSMUG1 compared with hUNG2. Thus, whereas pyrimidines having bulky substitutions at the 5-position do not have access to the hUNG2 active site, hSMUG1 is able to accommodate such substitutions at the 3-, 4-, and 5-positions.

Based on analysis of UDG activities from Ung−/− mice (23) and the low Km value (0.035 μM) of Xenopus SMUG1 (14), it was suggested that SMUG1 evolved to counteract the mutagenic effects of deaminated cytosines, and that the relative contribution of UNG2 to the repair of this lesion was reduced in mammalian cells (23). The data presented in Fig. 3 indicate that hUNG2 removes U from U-G mismatches even more efficiently than hSMUG1 and is thus a candidate enzyme to counteract cytosine deamination. To further analyze this, the reaction kinetics of hUNG2 and hSMUG1 were analyzed using oligonucleotides containing A,G, or no base opposite the substrate (U of HmU) and in the presence of Mg2+. (Table II). The results demonstrated that hUNG2 had a 300-fold higher kcat/Km than hSMUG1 against U-G mismatches, and that this was caused both by a lower Km and a higher kcat in the case of hUNG2. Furthermore, hUNG2 had a >100-fold higher kcat/Km than hSMUG1 against ssU. Although the results with short oligo-

![FIG. 2. Effects of pyrimidines on hUNG2 and hSMUG1. The activities of hUNG2 and hSMUG1 against [3H]dUMP-containing calf thymus DNA were analyzed in the presence of varying 19-mer oligonucleotides containing AP sites or normal bases. Filled triangles, duplex AP; filled inverted triangles, duplex AP,G; filled circles, single-stranded AP oligonucleotide; open inverted triangles, C,G control; open circles, single-stranded oligonucleotide containing C instead of an AP site. A, hUNG2 in the absence of Mg2+. B, hUNG2 in the presence of Mg2+.](Image 69x615 to 295x738)

![FIG. 3. Substrate specificities of hUNG2 and hSMUG1. Varying concentrations of hUNG2 or hSMUG1 were incubated for 30 min at 37°C with 5-32P-labeled oligonucleotides containing uracil, 5-FU, HmU, or εC. 1 ng of APE1 was included in all reactions. The upper bands observed after denaturing PAGE and phosphorimaging represent uncleaved 19-mer substrate, whereas the lower bands represent cleaved products. Extra bands observed below the APE1 cleavage products from FU and εC are caused by some base loss and cleavage during the final piperidine treatment.](Image 320x566 to 560x738)

**TABLE II**

| Enzyme | Substrate | Km (μM) | kcat (min⁻¹) | kcat/Km (min⁻¹ μM⁻¹) |
|--------|-----------|---------|--------------|-----------------------|
| hUNG2  | U:A-ds    | 0.7 ± 0.1 | 137 ± 4      | 187                   |
| hUNG2  | U:G-ds    | 0.4 ± 0.1 | 334 ± 14     | 750                   |
| hUNG2  | U:ss      | 2.2 ± 0.2 | 2758 ± 105   | 1258                  |
| hSMUG1 | U:A-ds    | 4.0 ± 0.9 | 3.1 ± 0.4    | 0.8                   |
| hSMUG1 | U:G-ds    | 1.3 ± 0.2 | 3.3 ± 0.2    | 2.5                   |
| hSMUG1 | U:ss      | 1.7 ± 0.3 | 17.5 ± 1.2   | 11                    |
| hSMUG1 | 5-HmU:A-ds| 4.6 ± 0.4 | 0.9 ± 0.03   | 0.2                   |
| hSMUG1 | 5-HmU-G-ds| 2.7 ± 0.3 | 2.4 ± 0.1    | 0.9                   |
| hSMUG1 | 5-HmU:ss  | 3.8 ± 0.5 | 5.9 ± 0.3    | 1.5                   |
This was also the case when EYFP was fused to the C-terminal end of hSMUG1 (data not shown). Furthermore, hSMUG1 appeared to be especially abundant in the nucleoli both in replicating and non-replicating cells. The physical localization of the two proteins indicates that both hUNG2 and hSMUG1 are available for repair of deaminated cytosines outside replication foci, in accordance with their substrate preferences. Only hUNG2, however, appears to have a function in postreplicative repair in replication foci. Conversely, only hSMUG1 is observed in nucleoli. The latter observation is intriguing, and may reflect a yet unrecognized function of hSMUG1 in nucleoli.

$hUNG2$ Is the Major Glycosylase for Removal of Both Deaminated Cytosine and Misincorporated Uracil in Nuclear Extracts—Nilsen et al. (23) recently provided evidence that SMUG1 represented a major glycosylase against U-G mismatches in ung-deficient mice. To analyze whether this was also so in human cells and to compare the hSMUG1 activity against oligonucleotides containing U:A and U:G, nuclear extracts from HaCaT, HeLa, and CX-1 cells were incubated with neutralizing antibodies to specifically inhibit hUNG2, hSMUG1, or both. In separate experiments the degree of inhibition and specificity of the anti-hUNG PU101 and anti-hSMUG1 PSM1 polyclonal antibodies were analyzed. Both antibodies proved to be specific, and essentially no cross-reactivity was observed with the two enzymes (Fig. 5A). The apparently higher concentration of antibody necessary to inhibit hSMUG1 was a result of the relatively high concentration of hSMUG1 needed to obtain measurable activity using $[^{3}H]dUMP$-calf thymus DNA as substrate. The latter concentration was not considered to pose a problem when neutralizing nuclear extracts, as the number of SMUG1 molecules per cell in the present cell lines appears to be considerably lower than UNG2. Whereas hUNG2 is easily detected by Western analysis of cell-free extracts, we were not able to detect hSMUG1 without previous concentration by immunoprecipitation (data not shown). Inhibition of the recombinant proteins by the PBS2-encoded inhibitor Ugi was also analyzed. Ugi proved to be a potent inhibitor of hUNG2. However, ~40% of the hSMUG1 activity was also inhibited at the concentrations of Ugi needed to fully inhibit hUNG2 (data not shown). Thus, to provide the highest specificity, neutralizing antibodies were used as inhibitors throughout the present study. The inhibited nuclear extracts were then incubated with $^{33}P$-labeled 19-mer oligonucleotides U141A and U141G (0.2 pmol) were incubated for 1 h at 37°C with nuclear extracts (5 μg of protein) that were preincubated in the absence or presence of neutralizing antibodies (0.5 μg) as indicated. The lower bands represent 9-mer cleavage products after uracil excision and piperidine cleavage.

$hUMP2$ and $hSMUG1$ in Base Excision Repair

**Fig. 4. Subnuclear localization of hUNG2 and hSMUG1.** HeLa cells were transiently or stably (ProA-UNG2-EYFP) transfected with the indicated constructs, and analyzed by laser confocal scanning microscopy as described under “Experimental Procedures.” The left column shows subnuclear localization pattern of hUNG2 and hSMUG1 in fusion with EYFP. Cells in early and middle columns can be identified by the distinct focal distribution of PCNA (middle column). Note that hUNG2 is largely excluded from nucleoli, whereas hSMUG1 accumulates in nucleoli.

**Fig. 5. Inhibition by neutralizing antibodies demonstrates relative contribution of hUNG2 and hSMUG1 activities in nuclear extracts.** A, recombinant hUNG2 or hSMUG1 were preincubated with or without neutralizing anti-UNG (PU101) or anti-SMUG1 (PSM1) prior to assay against $[^{3}H]dUMP$-labeled calf thymus DNA to monitor their neutralizing capacity and specificity. Open circles, hUNG2/PSM1; closed circles, hUNG2/PU101; open squares, hSMUG1/PU101; closed squares, hSMUG1/PSM1. B, $^{33}P$-labeled 19-mer oligonucleotides U141A and U141G (0.2 pmol) were incubated for 1 h at 37°C with nuclear extracts (5 μg of protein) that were preincubated in the absence or presence of neutralizing antibodies (0.5 μg) as indicated. The lower bands represent 9-mer cleavage products after uracil excision and piperidine cleavage.
UNG-neutralizing PU101 did not affect the amount of excised HmU, whereas preincubation with SMUG1-neutralizing PSM1 inhibited essentially all HmU glycosylase activity (Fig. 6A). Thus, SMUG1 likely represents the major HmU glycosylase activity in the nuclear extracts. Alternatively, a HmU glycosylase antigenically similar to SMUG1 may be present in human cells. To investigate the latter possibility, total protein was extracted from synchronized HaCaT cells at different times after release from serum starvation. hSMUG1 was immunoprecipitated using PSM1 covalently coupled to magnetic beads and subjected to SDS-PAGE and Western analysis. The lane at the left contains recombinant hSMUG. C, DNA synthesis in the cells harvested at the same time points as in B were monitored by [3H]thymidine pulse labeling.

In Vitro Short-patch BER of U:A Is Initiated by hUNG2, whereas Repair of U:G May Be Initiated by Alternative Mechanisms—To investigate whether UNG2 and SMUG1 from human cells were able to initiate BER of uracil in vitro, nuclear extracts from HeLa and CX-1 cells were assayed for short-patch BER activity using a closed circular plasmid substrate containing a single U:A or U:G at a defined position. The extracts were preincubated in the presence or absence of neutralizing anti-UNG (PU101) and/or anti-SMUG1 (PSM1) as indicated and the BER reaction allowed to proceed for 60 min. B, the degree of inhibition of the glycosylase step was analyzed using 50 bp of [3P]-labeled oligonucleotides corresponding to the region encompassing uracil in the plasmid substrates in A, and under otherwise identical conditions except that dNTPs were replaced by ddCTP or ddTTP to arrest polymerization and ligation. C, time-course BER assay using CX-1 nuclear extracts. The extracts were preincubated with neutralizing antibodies as in A, and reactions incubated for 10, 30, or 60 min. Ab, antibody.
identical experimental conditions. In all BER experiments above, at least 90% of the product resulted from short-patch repair incorporation of only one nucleotide, whereas less than 10% resulted from long-patch BER (data not shown). Thus, the non-inhibited U:G repair was likely not caused by nucleotide excision repair or mismatch repair. The latter was also verified by experiments using nuclear extracts from mismatch repair-deficient HCT116 colorectal cancer cells (ATCC CCL-247), in which U:G repair of gaps was also observed in the presence of both neutralizing antibodies (data not shown). Moreover, the short-patch DNA-repair process did not appear to be dependent on PCNA loading by replication factor C, because exclusion of ATP from the reactions had no effect on the steps prior to ligation (data not shown). A time-course BER experiment indicated, however, that the degree of inhibition by the neutralizing antibodies varied with the incubation time of the BER reactions. At short incubation times, preincubation with anti-UNG antibodies essentially abolished BER, whereas at prolonged incubation times the level of BER product in the anti-UNG reactions approached that of the uninhibited reaction (Fig. 7C). This indicates that repair of the U:G mismatch plasmid takes place by two distinct short-patch BER mechanisms. In the initial phase of the reaction, hUNG2-initiated short-patch BER dominates. In the late phase, initiation takes place by a yet unidentified mechanism, which is not detected using U:G oligonucleotides as substrate. The possible mechanisms of this alternative mode of repair will be discussed below.

**DISCUSSION**

By purifying the recombinant nuclear isoforms of hUNG2 and hSMUG1 to homogeneity, we are now, for the first time, able to carefully determine the kinetic parameters of hUNG2 and hSMUG1 and to compare their properties directly. The present biochemical data strongly suggest that UNG2 has a broader function than mere postreplicative repair of incorporated uracil, and likely is the major nuclear enzyme for repair of deaminated cytosine in both double-stranded and single-stranded DNA. Given the unsurpassed efficiency of hUNG2 compared with hSMUG1 to remove uracil, it is reasonable to believe that SMUG1 serves another primary function in higher eukaryotes. hSMUG1 shares many of the characteristics of hUNG2. There are, however, clear differences, such as markedly lower turnover number, strong binding to AP sites, broader substrate specificity, and the accumulation of hSMUG1 in nucleoli but not in replication foci. Notably, the activity of hSMUG1 against HmU is nearly as high as against U, indicating that this might be the primary substrate for hSMUG1 in vivo. This is also supported by the phylogenetic distribution of SMUG1, which is linked to the use by organisms of 5-methylcytosine as a mediator of gene expression (40). The present work shows that recombinant hSMUG1 has a substrate preference for HmU in the order ssHmU > HmU:G > HmU:A (Table II). For the corresponding U-containing substrates, somewhat higher activities are observed, although the order of preference is retained. This preference for U-containing substrates is also found by Boorstein and co-workers (15). More recently, however, a HmU-DNA glycosylase having distinct, but overlapping, substrate specificity compared with hSMUG1 was partially purified from HeLa cells (38). Like hSMUG1, this enzyme excised both U and HmU opposite A and G, with a preference for U. Likewise, the enzyme had a weak activity against 5-fluorouracil. However, no activity was observed against single-strand substrates or 3,N2-ethenocyto- sine. This activity may be the same as previously observed by Radany et al. (41) in human glioma cells, as the latter had no activity against G:T mismatches and was strictly specific for paired uracil with a preference for U:G. Interestingly, we identified a second EST derived from the SMUG1 gene by database search (NM_014311). This novel splice form is identical to SMUG1 in the 135 N-terminal amino acids, but utilizes a different reading frame of the SMUG1 3'-untranslated region to generate the 42 C-terminal amino acids. The biological function of this novel variant, if any, is not known. Two of three main motifs believed to be important for SMUG1 glycosylase activity (5), including the residues Asp-163 and Arg-243, are not present in the variant, suggesting that this protein may have activities distinct from SMUG1.

The finding that hUNG2 is a highly effective single-strand selective UDG in the presence of physiological concentrations of magnesium may indicate that this is an important function of hUNG2 in vivo. Transient single-strand regions occur frequently in chromatin both as a consequence of “breathing” in A:T-rich DNA, and in normal DNA metabolism such as replication, transcription, and recombination. Furthermore, because the rate of deamination of cytosine is more than 100-fold higher in ssDNA than in dsDNA (1), this would justify an efficient enzymatic scanning for uracil in ssDNA. This is substantiated by the finding that the mutation rate in Ung-deficient yeast cells was increased 2-fold by a high transcription rate (42). Efficient removal of deaminated cytosine from transcriptionally active ssDNA could also be important to avoid miscoding transcriptional and splicing of harmful proteins. The potential of generating such proteins was demonstrated for human UNG, as a single base pair substitution changed UNG from being a DNA repair enzyme to actually becoming a mutator enzyme (12). Notably, hUNG2 contains two RPA-binding motifs in the N-terminal regulatory domain (4), which may aid the recruitment to single-stranded regions of DNA.

In the present study we identified a BER-initiating activity that is apparently specific for long and/or circular U:G substrates, and that is not inhibited by neutralizing antibodies against hUNG or hSMUG1. Slow U:G-BER could in principle be initiated by the mismatch glycosylases TDG or MBD4. However, in the cell lines used in this study, hUNG2 and hSMUG1 represented all detectable UDG activity, even at high concentrations of magnesium. The activity of this novel variant, if any, is not known. Two of three main motifs believed to be important for SMUG1 glycosylase activity (5), including the residues Asp-163 and Arg-243, are not present in the variant, suggesting that this protein may have activities distinct from SMUG1.

As shown in Fig. 8, uracil and HmU in DNA may be present in different positions relative to a replication fork, and in addition the sequence context may vary. It seems likely that both the type of initiating UDG, as well as the BER subpathway in the subsequent steps, will depend on these factors. Our data indicate that the majority of HmU is excised by hSMUG1 outside of replication foci, and is thus likely processed by short-patch BER. Deaminated cytosine present in dsDNA prior to replication, e.g. in the G1-phase, may in principle be removed by any of the four identified human uracil-DNA glycosylases.
Most likely, hTDG and hMBD4 mainly function in CpG contexts and in ssDNA only, whereas hSMUG1 and hUNG2 may operate in any sequence context, albeit with varying efficiency (6, 44, 45). SMUG1 was recently suggested to be a major enzyme in repair of uracil in U-G mismatches (23). However, our data strongly suggest that, even in repair of U-G mispairs resulting from cytosine deamination, hUNG2 may be a major player. hUNG2 has at least as low a Km as hSMUG1, and is present in replication foci as well as in the nucleoplasm. However, hUNG2 is essentially excluded from nucleoli, whereas hSMUG1 accumulates in nucleoli, suggesting that hSMUG1 may have a specialized role in uracil repair in nucleoli. Among the uracil-DNA glycosylases, only hUNG2 specifically accumulates in the replication foci during S-phase, and all experimental evidence suggests that UNG2 has an important role in the removal of misincorporated uracil in replication foci.

UNG2 is by far the most efficient enzyme for removal of uracil in ssDNA. The lack of AP site re-binding by hUNG2 should not pose a problem in ssDNA outside of replication foci because the double-helical DNA conformation is likely restored to single-stranded region in front of the replication fork, hUNG2 probably excises uracil and leaves an AP site that may stall the replication fork and induce recombination or fork regression. Because AP endonucleases are highly double-strand-specific, the risk of creating a strand break (functionally a double-strand break) is very low. AP sites at the replication fork are most likely repaired either by BER subsequent to fork regression, or by recombination using information from the sister chromatid (which is now double-strand in this short region). Alternatively, AP sites are bypassed by translesion synthesis (TLS).

FIG. 8. Unified model for the occurrence and repair of uracil and HmU in replicating and non-replicating chromatins. The figure indicates the likely routes whereby uracil and HmU are introduced in DNA. Note that, when deaminated cytosines occur in the single-stranded region in front of the replication fork, hUNG2 probably excises uracil and leaves an AP site that may stall the replication fork and induce recombination or fork regression. Because AP endonucleases are highly double-strand-specific, the risk of creating a strand break (functionally a double-strand break) is very low. AP sites at the replication fork are most likely repaired either by BER subsequent to fork regression, or by recombination using information from the sister chromatid (which is now double-strand in this short region). Alternatively, AP sites are bypassed by translesion synthesis (TLS).
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