NEIL1 Is the Major DNA Glycosylase that Processes 5-Hydroxyuracil in the Proximity of a DNA Single-Strand Break

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ABSTRACT: 5-Hydroxyuracil (5-OHU) in DNA, arising during endogenous DNA damage and caused by ionizing radiation, is removed by the base excision repair pathway. However, in addition to base lesions, ionizing radiation also generates DNA single-strand breaks (SSBs). When these DNA lesions are located in the proximity of each other, this may result in a profound effect on both repair of the damaged base and the SSB. We therefore examined the repair of DNA substrates containing 5-OHU lesions in the proximity of the 3′-end of a SSB. We found that SSB repair by DNA ligase IIIα and DNA polymerase β is impaired by the presence of the nearby 5-OHU lesion, indicating the requirement for a DNA glycosylase which would be able to remove 5-OHU before SSB repair. Subsequently, we found that although both SMUG1 and NEIL1 are able to excise 5-OHU lesions located in the proximity of the 3′-end of a DNA SSB, NEIL1 is more efficient in the repair of these DNA lesions.

Endogenous oxidative metabolism and exogenous agents, such as ionizing radiation, can generate reactive oxygen species (ROS)[1] that attack DNA to produce a plethora of lesions, such as base deamination, oxidized bases, and DNA strand breaks (*1*). For example, the oxidation of cytosine to the unstable lesion cytosine glycol and subsequent deamination generates 5-hydroxyuracil (5-OHU) lesions that are commonly observed following ionizing radiation and are premutagenic lesions giving rise to C to T transitions (*2*, *3*). Furthermore, ionizing radiation can also produce ROS at high local concentrations, giving rise to DNA lesions in the proximity of each other (*4*). These can represent a challenge to the DNA repair machinery and thus require more complex and temporally regulated steps in the repair process. Indeed, such "complex" lesions have been shown to delay repair and generate DNA double-strand breaks and mutations (*5*−*7*).

DNA lesions in the proximity of each other have been shown to be repaired by enzymes of the base excision repair (BER) pathway (*8*−*10*). BER, the major pathway for the repair of DNA base lesions, is initiated by a DNA glycosylase that excises the damaged base (*11*). The resulting AP site is then cleaved by AP endonuclease (APE1) and the strand break repaired by DNA polymerase β (Pol β) and XRCC1-DNA ligase IIIα heterodimer, culminating in "short-patch" BER (*12*). In human cells, 5-OHU is a major substrate for both the endonuclease III homologue (NTH1) and endonuclease VIII (Nei)-like protein 1 (NEIL1) (*13*). Surprisingly, we have previously found that NTH1 very inefficiently excises 5-OHU located in the proximity of a DNA SSB (*14*). We further demonstrated that these lesions are in fact excised by NEIL1 (*14*). However, UNG2 and SMUG1, which are the major uracil-DNA glycosylases (*15*−*17*), have also been reported to excise 5-OHU (*18*, *19*), yet little is known about the relative activity and biological significance of these enzymes on 5-OHU in the proximity of a DNA SSB.

In this work, we further examine the effect of 5-OHU in the proximity of the 3′-end of a DNA SSB on the repair of the SSB itself and also compare the activities of the major DNA glycosylases involved in excision of the damaged base. We discovered that the presence of a nearby 5-OHU lesion drastically inhibits repair of the SSB, and furthermore, we demonstrate that NEIL1 is the major enzyme responsible for the excision of 5-OHU when present near the 3′-end of a DNA SSB while SMUG1, which is much less active, may act as a backup repair activity.

MATERIALS AND METHODS

Materials. Synthetic oligodeoxyribonucleotides were purchased from Eurogentec and purified on a 20% polyacrylamide gel. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Perkin-Elmer Life Sciences. Histidine-tagged human DNA polymerase β, polynucleotide kinase, and DNA ligase IIIα were purified on a Ni^{2+}-charged His-Bind resin (Novagen) as described by the manufacturer. Full-length native mouse NEIL1 was kindly provided by D. Zharkov, and NEIL1 antibodies were kindly provided by T. Rosenquist. UNG2, SMUG1, and antibodies against SMUG1 were prepared as described previously (*17*, *20*).

Substrate Labeling. Oligonucleotides were 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, and

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[1] Abbreviations: ROS, reactive oxygen species; BER, base excision repair; APE1, apurinic/apyrimidinic endonuclease 1; Pol β, DNA polymerase β; Lig III, DNA ligase IIIα; NEIL1, endonuclease VIII-like protein 1; PNK, polynucleotide kinase; 5-OHU, 5-hydroxyuracil; SSB, single-strand break; UNG2, uracil DNA glycosylase 2; SMUG1, single-strand selective monofunctional uracil DNA glycosylase 1.

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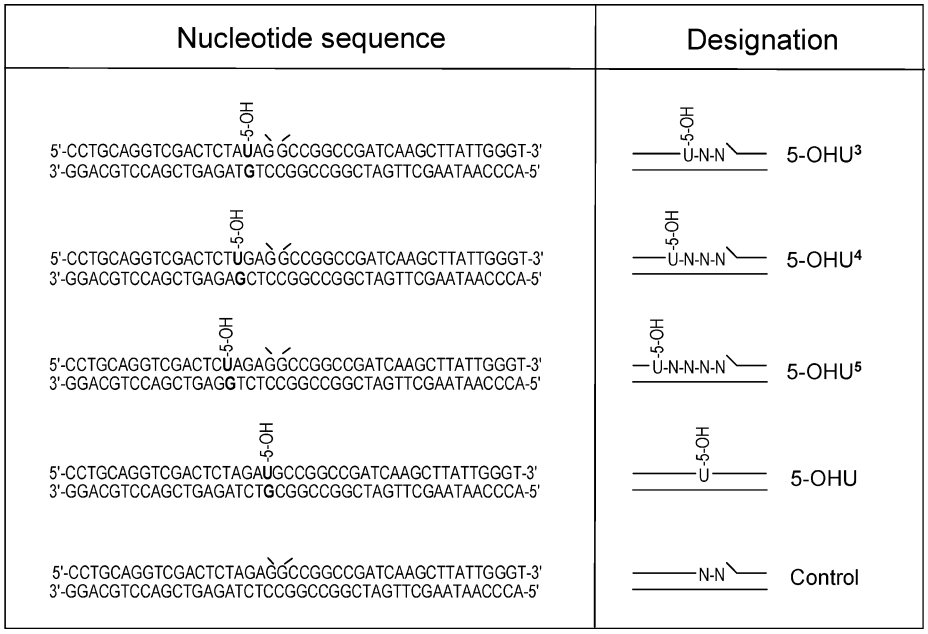

FIGURE 1: Structures of oligonucleotides that were used. Oligonucleotides (20-mer) containing 5-OHU were 5′-end-labeled, and a 24-mer adjacent oligonucleotide was added and annealed to the corresponding complementary strand to generate substrates containing 5-OHU located three to five nucleotides from a DNA single-strand break. Substrates containing 5′-end-labeled isolated 5-OHU and those containing 5′-end-labeled single-strand breaks only were also used as controls. N refers to a normal base.

unincorporated label was removed on a BioSpin P-6 spin column (Bio-Rad). To prepare the substrates, the labeled oligonucleotides were annealed to the relevant oligonucleotides shown in Figure 1 at 90 °C for 3−5 min followed by slow cooling to room temperature.

Repair Assays. Assays using purified proteins contained 300 fmol of oligonucleotide per reaction mixture in 10 μL of reaction buffer containing 50 mM Hepes-KOH (pH 7.8), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 1.5 mM DTT, 8.5% glycerol, and 100 μg/mL BSA. DNA glycosylase reactions were performed in the presence of 150 fmol of APE1; primer extension reactions also included dCTP, dATP, dGTP, and TTP (20 μM each), and ligation reaction mixtures were supplemented with 2 mM ATP. All reaction mixtures were incubated for 20 min at 37 °C; 10 μL of formamide loading dye (95% formamide, 0.02% xylene cyanol, and 0.02% bromophenol blue) was added, and the samples were heated to 95 °C for 5 min. Products were subsequently analyzed by 20% denaturing polyacrylamide gel electrophoresis and gels exposed to storage phosphor screens at 4 °C prior to analysis by phosphorimaging. To calculate enzyme kinetic parameters, SMUG1 or NEIL1 was incubated with varying amounts of substrate (30−600 fmol; seven points in total) in the presence of APE1 (150 fmol) for 10 min at 37 °C. The gels were quantified using Quantity One to calculate the percentage cleavage of the substrate, and the velocity of the reaction (nanomolar per minute) was subsequently determined and used to produce Lineweaver−Burk plots. At least three independent experiments were performed.

Electrophoretic Mobility Shift Assay (EMSA). Assays contained 300 fmol of oligonucleotide and the indicated amount of either SMUG1 or NEIL1 per reaction mixture in 20 μL of buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM KCl, 3 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 20 μg/mL BSA, 7.5% glycerol, and 0.1% Triton X-100 and were conducted for 15 min on ice. Five microliters of loading dye (30% glycerol, 0.25% xylene cyanol, and 0.25% bromophenol blue) was added, and the samples were analyzed on a 12% nondenaturing polyacrylamide gel at 4 °C in 0.5× TBE. The gels were dried and exposed to storage phosphor screens prior to being analyzed by phosphorimaging.

Quantitative Western Blot Analysis. Whole cell extracts were prepared from HeLa cell pellets (Paragon) by the method of Manley et al. (*21*) and dialyzed overnight against buffer containing 25 mM Hepes-KOH (pH 7.9), 100 mM KCl, 12 mM $MgCl_2$, 0.1 mM EDTA, 17% glycerol, and 2 mM DTT prior to being stored at −80 °C. Varying amounts of extracts and either NEIL1 or SMUG1 protein were separated by 10% SDS−PAGE, transferred to Immobilon-FL membranes (Millipore), and immunoblotted with either NEIL1 or SMUG1 antibodies (see Materials). Blots were visualized and quantified using the Odyssey image analysis system (Li-cor Biosciences).

RESULTS

DNA Single-Strand Breaks Containing 3′-End Proximal 5-OHU Lesions Are Poorly Processed by DNA Ligase and DNA Polymerase. To examine the repair of DNA substrates containing 5-OHU lesions located near a DNA SSB, oligonucleotide substrates containing 5-OHU in different positions proximal to the 3′-end of a DNA SSB were generated (Figure 1). The 5-OHU lesion-containing strand was 5′-end-labeled with ^{32}P so the excision of the modified base could be monitored and also so the repair of the SSB could be examined. First, we examined whether the presence of the 5-OHU lesion can impact repair of the SSB by other BER enzymes, namely, DNA ligase IIIα (Lig III) and DNA polymerase β (Pol β). We found that there was an approximate 2−3-fold reduction in Lig III activity with all three substrates containing 5-OHU in the proximity of the 3′-end of a SSB (Figure 2A, second through fourth panels) in comparison to the nicked substrate only (Figure 2A, first

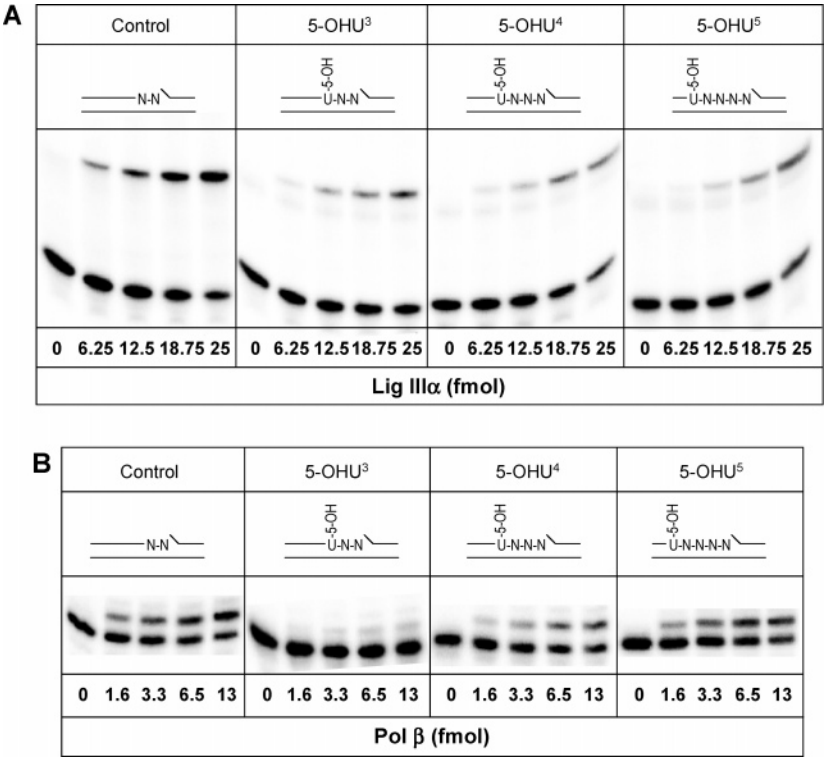

FIGURE 2: Comparison of DNA ligase IIIα and DNA polymerase β activities on DNA substrates containing 5-OHU in the proximity of a DNA single-strand break. Oligonucleotide substrates (0.3 pmol), shown at the top of the panel, were incubated with increasing concentrations of (A) DNA ligase IIIα (0−25 fmol) or (B) DNA polymerase β (0−13 fmol) for 20 min at 37 °C prior to the addition of formamide loading dye. An aliquot was analyzed by 20% denaturing polyacrylamide gel electrophoresis and phosphorimaging.

panel). Conversely, one nucleotide gap filling by Pol β was found to be almost completely blocked when 5-OHU^3 was used as a substrate (Figure 2B, second panel). There was also an approximate 3-fold reduction in the level of incorporation when 5-OHU^4 was used (Figure 2B, third panel) in comparison to a gapped substrate in the absence of a nearby 5-OHU lesion (Figure 2B, first panel). However, full Pol β gap filling activity appeared to be restored on 5-OHU^5 (Figure 2B, fourth panel). This demonstrates that both DNA ligase and DNA polymerase activities are compromised by the presence and position of 5-OHU located near the 3′-end of a DNA SSB and suggests that this lesion should be processed by a DNA glycosylase prior to repair of the SSB.

Excision of 5-OHU Located Near the 3′-End of a DNA Single-Strand Break by UNG2 and SMUG1. We have recently shown that 5-OHU lesions in the proximity of the 3′-end of a DNA SSB are resistant to cleavage by NTH1 but are excised by NEIL1 (*14*). However, UNG2 and SMUG1 have also been reported to excise 5-OHU with varying degrees of specificity (*18*, *19*), although the effect of a neighboring SSB on their activities has not been investigated. Therefore, we examined the DNA glycosylase activities of UNG2 and SMUG1 on 5-OHU located near the 3′-end of a DNA SSB. Using approximately equimolar amounts of UNG2 and SMUG1 in the reaction, we found that UNG2 had a very low activity toward 5-OHU whether isolated in DNA (Figure 3, fourth panel) or in the proximity of the 3′-end of a SSB, at the concentration that was tested (Figure 3, first through third panels). However, SMUG1 readily excised 5-OHU when it was isolated in DNA (Figure 3, fourth panel) and also near the 3′-end of a SSB (Figure 3, first through third panels) with varying degrees of success. Interestingly, the DNA glycosylase activity of SMUG1 toward 5-OHU appeared to increase the farther it was from the strand break: 5-OHU^5 > 5-OHU^4 > 5-OHU^3 = 5-OHU (Figure 3). This demonstrates a potential role for SMUG1 during the repair of 5-OHU located near a DNA SSB.

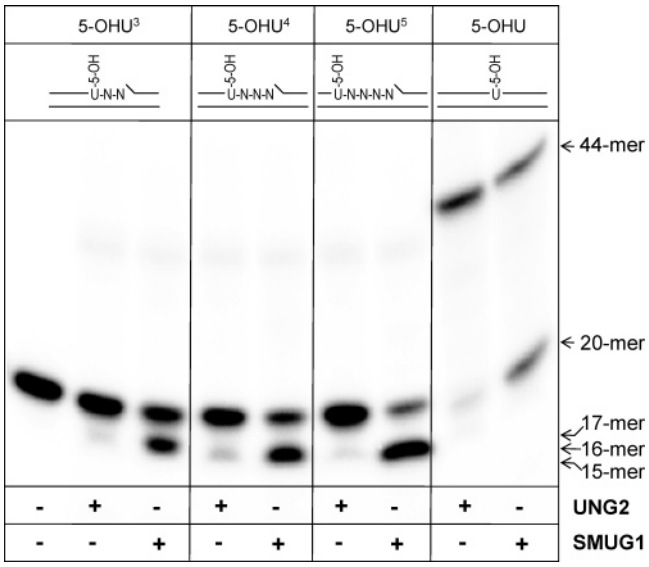

FIGURE 3: Comparison of the excision of 5-OHU located near a DNA single-strand break by UNG2 and SMUG1. Oligonucleotide substrates (0.3 pmol), shown at the top of each panel, were incubated with either UNG2 (2.9 pmol) or SMUG1 (3.3 pmol) in the presence of APE1 (150 fmol) for 20 min at 37 °C, and formamide loading dye was added. An aliquot was analyzed by 20% denaturing polyacrylamide gel electrophoresis and phosphorimaging.

Table 1: Kinetic Constants for Excision of 5-OHU from Oligonucleotide Substrates by SMUG1 and NEIL1[a]

enzyme	substrate	K_m (nM)	k_{cat} ($\times 10^3$ min^{-1})	K_{cat}/K_m ($\times 10^3$ min^{-1} nM^{-1})
NEIL1	5OHU·G	36.2 ± 8.4	541.2 ± 224.0	14.5 ± 3.4
NEIL1	5OHU^3·G	27.0 ± 15.7	406.8 ± 149.7	16.5 ± 3.9
NEIL1	5OHU^4·G	21.5 ± 9.8	345.1 ± 92.5	17.8 ± 6.3
SMUG1	5OHU·G	53.2 ± 7.3	20.4 ± 6.2	0.4 ± 0.2
SMUG1	5OHU^3·G	20.1 ± 7.3	9.0 ± 5.2	0.5 ± 0.3
SMUG1	5OHU^4·G	64.1 ± 16.7	128.6 ± 79.7	2.4 ± 2.0

[a] SMUG1 and NEIL1 were incubated with increasing concentrations of the relevant oligonucleotide substrate (30−600 fmol; seven points in total) for 10 min at 37 °C in the presence of APE1 (150 fmol). Formamide loading dye was added, and an aliquot was analyzed by 20% denaturing polyacrylamide gel electrophoresis and phosphorimaging. Kinetic parameters were determined using Lineweaver−Burk plots, and the averages from at least three independent experiments are given.

Comparison of Excision of 5-OHU Located Near the 3′-End of a DNA Single-Strand Break by NEIL1 and SMUG1. As we have previously observed NEIL1 activity on 5-OHU located near the 3′-end of a DNA SSB (*14*), we compared it with SMUG1 activity by calculating kinetic parameters for both enzymes (Table 1). We found that the rate of turnover (K_{cat}) of NEIL1 against 5-OHU isolated in DNA was approximately 25-fold higher than that observed with SMUG1, which also had a slightly higher (1.5-fold) K_m value demonstrating that NEIL1 has a much greater activity toward isolated 5-OHU lesions in DNA than SMUG1. Interestingly, the introduction of a DNA SSB positioned as a third (5-OHU^3) or fourth (5-OHU^4) nucleotide 3′ to the lesion only partially decreased the average K_{cat} values observed for NEIL1, although they are all within experimental error; however, the specificity constants (K_{cat}/K_m) demonstrate that they are still good substrates for the enzyme. However, the effect of a 3′-proximal SSB on the cleavage of 5-OHU by SMUG1 was more dramatic. Using 5-OHU^3 in comparison to 5-OHU alone in DNA, the K_{cat} of SMUG1 decreased approximately 2-fold, and using a gel shift assay, we also found that NEIL1 has a higher affinity for this substrate than SMUG1, indicating that NEIL1 may be the major enzyme involved in its repair (Figure 4A). In contrast, the excision of 5-OHU^4 by SMUG1 was very efficient compared to that with 5-OHU alone, as demonstrated by approximately 6-fold increases in k_{cat} and K_m/k_{cat} values, demonstrating that the presence of a nearby SSB increases the substrate specificity of the enzyme. This was also apparent from Figure 3, where the activity of SMUG1 appeared to increase with an increase in the distance from the SSB (5-OHU^4 and 5-OHU^5) over and above that observed with 5-OHU alone. When the enzymatic activity for 5-OHU^4 was compared, NEIL1 has K_{cat} and K_m values approximately 3-fold higher and 3-fold lower than those of SMUG1, respectively, indicating the possibility that NEIL1 is the major enzyme for excision of 5-OHU in this position relative to the SSB.

We subsequently calculated the relative levels of both NEIL1 and SMUG1 enzymes in human HeLa whole cell extracts using quantitative Western blotting (Figure 4B). We found that NEIL1 and SMUG1 are present within human cell extracts at approximately 13.9 and 12.0 pmol/mg of protein, respectively. As NEIL1 also had the greater DNA glycosylase activity toward 5-OHU lesions, this further demonstrates that NEIL1 is the major enzyme for the excision of 5-OHU in the proximity of the 3′-end of a DNA SSB, although SMUG1 can act as a backup repair enzyme.

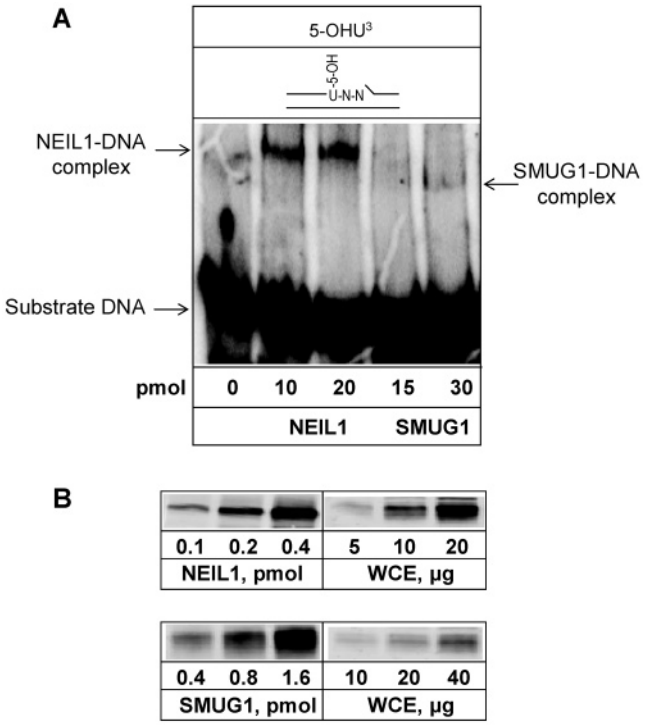

FIGURE 4: NEIL1 has a higher affinity for the 5-OHU^3 substrate and quantification of NEIL1 and SMUG1 in HeLa whole cell extracts. (A) An oligonucleotide substrate (5-OHU^3, 0.3 pmol) was incubated with 0−30 pmol of either SMUG1 or NEIL1 on ice for 15 min prior to separation by 12% nondenaturing gel electrophoresis at 4 °C. The gel was dried and exposed to storage phosphor screens at 4 °C prior to being analyzed by phosphorimaging. (B) Varying amounts of HeLa whole cell extract (5−40 μg) and either NEIL1 (0.1−0.6 pmol) or SMUG1 (0.4−3.4 pmol) protein were separated via 10% SDS−PAGE, transferred to PVDF membranes, and immunoblotted with either NEIL1 or SMUG1 antibodies. Blots were visualized and quantified using the Odyssey image analysis system.

Repair of 5-OHU Located Near the 3′-End of a DNA Single-Strand Break with Purified BER Proteins. As we discovered that NEIL1 has a greater activity toward 5-OHU when placed near the 3′-end of a DNA SSB than SMUG1, we reconstituted the repair of 5-OHU^3 using NEIL1 and other purified BER proteins. Incubation of a 5′-labeled substrate containing 5-OHU^3 (Figure 5A) with NEIL1 generates a 17-mer-labeled oligonucleotide (Figure 5B, lane 2) containing a three-nucleotide gap with a 3′-phosphate moiety that requires processing by polynucleotide kinase (PNK) to make the ends amenable to Pol β activity. After addition of PNK and cleavage of the 3′-phosphate moiety, Pol β fills the three-nucleotide gap, generating a 20-mer-labeled oligonucleotide that is ready for ligation (Figure 5B, lane 3). Furthermore, only a combination of all components including NEIL1, PNK, Pol β, and Lig III results in a full-length repaired product (Figure 5, lane 4, 44-mer). However, in the absence of either Pol β or PNK (Figure 5, lanes 5 and 6), very little full-length repaired product is observed, demonstrating that NEIL1 excises the DNA lesion prior to DNA ligase IIIα activity.

DISCUSSION

Endogenous oxidative metabolism or exogenous agents such as ionizing radiation may generate DNA lesions, such

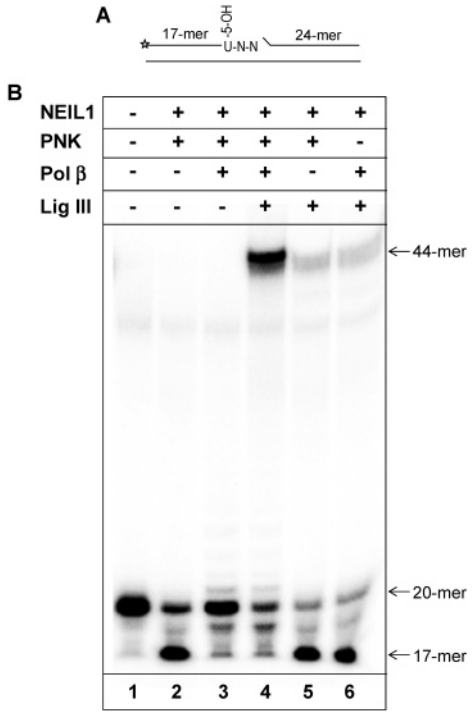

FIGURE 5: Reconstitution of repair of the 5-OHU[3] substrate with NEIL1 and purified BER proteins. (A) Structure of the oligonucleotide substrate (5-OHU[3]) used in the reactions in which the oligonucleotide containing 5-OHU (20-mer) was 5′-end-labeled (indicated with a star), and a 24-mer adjacent oligonucleotide was added and annealed to the corresponding complementary strand to generate a substrate containing 5-OHU located three nucleotides from a DNA single-strand break. (B) The substrate (5-OHU[3], 0.3 pmol) was incubated with either NEIL1 (0.7 pmol), PNK (17.5 fmol), DNA polymerase β (50 fmol), or DNA ligase IIIα (40 fmol) for 20 min at 37 °C, and the reaction was terminated by addition of formamide loading dye. An aliquot was analyzed by 20% denaturing polyacrylamide gel electrophoresis and phosphorimaging.

as 8-oxoguanine, 5-OHU, and many others, in the proximity of a DNA SSB. The presence of these DNA lesions may delay or block SSB repair that may subsequently cause genome instability and therefore represent a threat to cell survival. Previously (*14*), as well as in this study (data not shown), we observed that base modifications in the first two nucleotides at the 3′-end next to the SSB are not excised by DNA glycosylases. However, we and others have recently demonstrated that APE1 is the major activity excising 3′-end-damaged DNA bases in human cell extracts (*22*, *23*), and we have also found that DNA polymerase δ is the major enzyme capable of excising DNA lesions located as a second nucleotide from the 3′-end of a DNA SSB (Parsons et al., *Nucl. Acids Res.*, in press).

In this study, we focused on the repair of SSBs with 5-OHU located farther from the 3′-end. We found that the repair of a SSB in the presence of a nearby 5-OHU lesion by DNA ligase IIIα and DNA polymerase β, the major BER and SSB repair enzymes in human cells, is compromised. This indicates that the 5-OHU lesion should be excised by a DNA glycosylase prior to the repair of the nearby SSB that is deemed relatively inefficient. NEIL1 has recently been shown to have the unique ability to excise 5-OHU located in the proximity of a SSB, which is inefficiently cleaved by NTH1 (*14*). Our study now reveals that SMUG1 is also capable of excising 5-OHU lesions located near a DNA SSB. Interestingly, the activity of SMUG1 against 5-OHU lesions appeared to increase the farther it was from the SSB, indicating a structure specific preference for excision of 5-OHU by SMUG1. However, by calculating enzyme kinetic parameters and by quantitative Western blotting, we confirmed that NEIL1 is the major known DNA glycosylase active against 3′-end proximal 5-OHU lesions while SMUG1 may act as a backup repair activity. Furthermore, reconstitution of the repair of 5-OHU near the 3′-end of a DNA SSB using purified BER enzymes demonstrated that repair of such lesions is initiated by NEIL1, rather than through direct ligation by a DNA ligase. NEIL1 processing of the lesion generates a 3′-phosphate that requires PNK activity prior to gap filling and ligation, as previously shown (*24*).

Interestingly, a 10-fold increase in the level of spontaneous mutations at cytosine residues has been reported in cells deficient in both UNG2 and SMUG1 (*25*). These cells were also hypersensitive to ionizing radiation, and it suggests that a cytotoxic lesion is induced that is excised by both proteins and not by any other DNA repair enzymes. NEIL1 knockdown cells have also been found to be sensitive to γ-irradiation (*26*) that is known to induce lesions containing a combination of DNA strand breaks and damaged bases (*27*), including 5-OHU. Our study indicates that in the case of SMUG1 knockdown, 5-OHU lesions in the proximity of a DNA SSB may not be the major cytotoxic lesions, as these are repaired independently by SMUG1 and NEIL1. However, the possibility that the requirement for DNA glycosylases may be more complex in vivo where DNA is found in chromatin cannot be excluded. It would be interesting to examine whether a SMUG1 and NEIL1 deficient cell line is hypersensitive to the effects of ionizing radiation compared to cells lacking the individual proteins that may further demonstrate the importance of excising 3′-proximal 5-OHU lesions.

In summary, our results suggest that NEIL1 is the major known DNA glycosylase involved in the excision of 5-OHU in the proximity of the 3′-end of a DNA SSB, while SMUG1 may act as a backup repair activity.

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