Preferential Repair of Oxidized Base Damage in the Transcribed Genes of Mammalian Cells

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Preferential repair of bulky DNA adducts from the transcribed genes via nucleotide excision repair is well characterized in mammalian cells. However, definitive evidence is lacking for similar repair of oxidized bases, the major endogenous DNA lesions. Here we show that the oxidized base-specific human DNA glycosylase NEIL2 associates with RNA polymerase II and the transcriptional regulator heterogenous nuclear ribonucleoprotein-U (hnRNP-U), both in vitro and in cells. NEIL2 immunocomplexes from cells preferentially repaired the mutagenic cytosine oxidation product 5-hydroxyuracil in the transcribed strand. In a reconstituted system, we also observed NEIL2-initiated transcription-dependent base excision repair of 5-hydroxyuracil in the transcribed strand, with hnRNP-U playing a critical role. Chromatin immunoprecipitation/reimmunoprecipitation studies showed association of NEIL2, RNA polymerase II, and hnRNP-U on transcribed but not on transcriptionally silent genes. Furthermore, NEIL2-depleted cells accumulated more DNA damage in active than in silent genes. These results strongly support the preferential role of NEIL2 in repairing oxidized bases in the transcribed genes of mammalian cells.

Reactive oxygen species leading to oxidatively modified DNA bases have been implicated in a wide variety of pathophysiological states, including aging, arthritis, tumor induction and promotion, and atherosclerosis (1, 2). Oxidized base lesions in DNA are repaired via a highly conserved base excision repair (BER) pathway (3, 4) that is initiated with excision of the damaged base by DNA glycosylases, followed by DNA strand cleavage at the lesion site. The previously characterized oxidized base-specific mammalian DNA glycosylases include NTH1 (endonuclease III homolog 1; the human ortholog of Escherichia coli Nth), which primarily excises damaged pyrimidines, and OGG1 (8-oxoguanine-DNA glycosylase 1; the functional counterpart of E. coli Fpg), which repairs purine-derived lesions, including ring-opened formamidopyrimidine (Fapy G) (5, 6). Surprisingly, mouse nullizygous for Ogg1 or Nth1 have no strong phenotype and do not show significantly increased tumor incidence (7–9). Furthermore, Nth1-null mouse embryonic fibroblasts do not show increased sensitivity to oxidative stress (8), and a modest excision activity for the ring-opened oxidation product of G (Fapy G) was observed in Ogg1-null cells (7). Taken together, these results suggested that additional repair enzymes and/or pathways exist for the repair of oxidative base damage in the mammalian genome.

We and others have identified three orthologs of Nei/Fpg (oxidized base-specific E. coli DNA glycosylases) in the human genome data base, which we named NEIL1 to -3 (for Nei-like 1–3) (8, 10–14). It has been shown that NEILs have overlapping substrate specificities and uniquely excise oxidized bases from base-unpaired sequences in DNA that mimic the DNA bubbles associated with transcription and/or replication (15). Although oxidative damage is likely to be distributed throughout the genome, repair of transcriptionally active sequences, which constitute only a small fraction of the mammalian genome, is obviously more important than that of the rest of the genome in terminally differentiated, non-dividing cells that constitute the bulk of mammalian adult tissues. Preferential repair of bulky adducts in the transcribed strand of active genes via the nucleotide excision repair (NER) pathway, named transcription-coupled NER (TC-NER), has been well characterized (16–18). The existence of a similar transcription-coupled repair process for preferential removal of oxidized bases has been suggested (19, 20), although neither the precise nature of the proposed pathway nor the responsible glycosylase has been identified. Here we present experimental results that strongly support NEIL2-initiated transcribed gene-specific repair of oxidized bases in mammalian cells.

**EXPERIMENTAL PROCEDURES**

Purification and Characterization of Proteins—Recombinant NEIL2, PNK, Pol β, and Lig IIIα were purified to homo-
generation from *E. coli* as described earlier (11, 21, 22). We expressed His-tagged hnRNP-U in BL-21 Codon-Plus *E. coli* (Stratagene) and purified it from the cell extract by affinity chromatography on an Ni²⁺-NTA-agarose column (Qiagen). It was further purified to homogeneity by elution from Q-Sepharose (Amersham Biosciences) using a 100–750 mM salt gradient. The most purified fraction was eluted at 400 mM NaCl and stored after dialysis in PBS (pH 7.4) containing 450 mM NaCl, 50% glycerol, and 1 mM DTT. For most biochemical studies except for Western analysis, purified NEIL2 and hnRNPU from insect cells were used. Full-length NEIL2 with C-terminal (11) and hnRNPU with N-terminal His tag were expressed and purified from Sf9 insect cells. The full-length hnRNPU (residues 1–824) with an N-terminal His tag was amplified by PCR (using as the forward primer 5’-CCGGAA-TTCACCATGCATCATCATCATCATCATATGAGTTCC-3’ and as the reverse primer 5’-CCCCGGAAGCTTTCAATAATATCCTTGATATGCATGCTG-3’) and subcloned into pFastBacDual (Invitrogen) between the EcoRI and HindIII sites. The recombinant bacmid was expressed His-tagged hnRNP-U (soluble extract from 1 liter of Sf9 cells) was examined by Coomassie Blue staining after imidazole. HnRNP-U was further purified by chromatography on Q-Sepharose (Amersham Biosciences) using a 100–750 mM salt gradient. HnRNP-U was further purified by chromatography on Q-Sepharose and then dialyzed in PBS containing 300 mM NaCl, 50% glycerol, and 1 mM DTT and stored at −20 °C. The RNA polymerase II (RNAP II) basal transcription kit was purified His-tagged hnRNPU (soluble extract from 1 liter of Sf9 cells) was bound to Ni²⁺-NTA resin (Qiagen) at 4 °C for 1 h. After extensive washing with 25 mM Tris-HCl (pH 7.5), 500 mM NaCl containing 20 mM imidazole, the bound protein was eluted with the same buffer except that it contained 250 mM imidazole. HnRNPU was further purified by chromatography on Q-Sepharose and then dialyzed in PBS containing 300 mM NaCl, 50% glycerol, and 1 mM DTT and stored at −20 °C. The RNA polymerase II (RNAP II) basal transcription kit was purchased from ProteinOne (Bethesda, MD) and included purified HeLa cell RNAP II and the general transcription factors (26). The immunoprecipitates from the lysates with M2 anti-FLAG antibody (Sigma) were washed extensively with cold TBS (50 mM Tris-HCl, pH 7.5, 200 mM NaCl) containing 1 mM EDTA, 1% Triton X-100, and 10% glycerol and tested for the presence of RNAP II, hnRNPU, and other proteins in the complex using appropriate Abs. The immunoprecipitates from both control and NEIL2-FLAG-transfected cells were also employed in a large scale affinity pull-down (25) and eluted stepwise with 1 ml each of 25 mM Tris-HCl, pH 7.5, containing 250, 350, or 500 mM NaCl. The elutes were analyzed by SDS-PAGE after acetone precipitation, and protein bands that were specifically present in the NEIL2-FLAG immunoprecipitate and not in the control immunoprecipitate were subjected to mass spectroscopic identification in the University of Texas Medical Branch Biomolecular Resource Facility.

Far Western Analysis—Far Western analysis was carried out with hnRNPU and other purified proteins (used as controls) as described previously (27, 28). Briefly, purified proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose membranes (28). After two 5-min incubations in denaturation buffer (6 M guanidine HCl in PBS) at 4 °C, the membranes were incubated six times for 10 min each in 1:1 serial dilutions of denaturation buffer in PBS plus 1 mM DTT. The membranes were blocked with PBS containing 0.5% Tween 20 and 5% nonfat dry milk for 45 min at 20 °C. After two washes in PBS containing Tween 20 (0.5%) and nonfat dry milk, the blots were incubated for 3 h at 4 °C with NEIL2 (10 pmol/ml) in PBS containing Tween 20 (0.5%) and nonfat dry milk containing 250, 500, and 1000 µg/ml zeoocin. After 3 weeks, individual zeoocin-resistant clones were transferred into 6-well plates. Surviving clones were expanded, and the expression of FLAG-NEIL2 was tested; only the clones with a low level of expression were used in this study. To maintain the stable transfec
tant clones, a zeocin concentration of 200 µg/ml was maintained throughout.

Generation of a Stable NEIL2-depleted Cell Line—We used a recently described system (24) to generate lentiviruses expressing shRNAs to deplete NEIL2 in HEK293 cells. Briefly, oligonucleotides (shRNA-C, 5’-gcgcttgtgagaaat-3’; shRNA-N2, 5’-tcagcaggttgtaacagca-3’) were subcloned into the pENTR/pTRE + (430-1) vector, which was recombined with the pLenti X2 Puro (w16-1) vector. To produce lentivi
ruses, the pLenti X2 Puro/pTRE-shRNA-N2 and pLenti X2 Puro/pTRE-shRNA-C plasmids were transfected with packag
ng plasmids in 293FT cells (24). Lentiviruses were harvested 48 h post-transfection and filtered through a 0.45-µm syringe filter. Transduction of the HEK293 cells stably expressing NEIL2-FLAG was done as described earlier (24), and the transduced cells were selected 48 h post-transduction with 1.0 µg/ml puromycin. Depletion of NEIL2-FLAG was monitored by Western blotting using an anti-FLAG Ab.

Analysis of NEIL2-associated Proteins—Gastric epithelial cells (AGS) stably transfected either with NEIL2-FLAG or empty FLAG-vector as a control or normal AGS cells were washed with PBS and lysed in cold lysis buffer as described earlier (25). The cell lysates were then treated with benzoylase or micrococcal nuclease to remove all of the nucleic acids (26). The immunoprecipitates from the lysates with M2 anti-FLAG antibody (Sigma) were washed extensively with cold TBS (50 mM Tris-HCl, pH 7.5, 200 mM NaCl) containing 1 mM EDTA, 1% Triton X-100, and 10% glycerol and tested for the presence of RNAP II, hnRNPU, and other proteins in the complex using appropriate Abs. The immunoprecipitates from both control and NEIL2-FLAG-transfected cells were also employed in a large scale affinity pull-down (25) and eluted stepwise with 1 ml each of 25 mM Tris-HCl, pH 7.5, containing 250, 350, or 500 mM NaCl. The eluates were analyzed by SDS-PAGE after acetone precipitation, and protein bands that were specifically present in the NEIL2-FLAG imm
unoprecipitate and not in the control immunoprecipitate were subjected to mass spectroscopic identification in the University of Texas Medical Branch Biomolecular Resource Facility.

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**Antibodies**—The synthetic peptide WVNDFSRAKKA-NKRGDWC, corresponding to the deduced amino acid residues 142–158 of human NEIL2, was coupled to keyhole limpet hemocyanin and used to raise polyclonal antisera in rabbits (Biomolecular Resource Facility Core, University of Texas Medical Branch). The Ab was affinity-purified from crude antiserum by adsorption to and elution from the above synthetic peptide on CNBr-activated Sepharose 4B (GE Healthcare) and was used for our studies. Commercial Abs used were anti-FLAG M2 (Sigma); RNAP II (N20, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)); H-5 RNAP II (Covance); and hnRNPU, TFIIH, and TFIIIF (Bethyl Laboratories).

**Generation of a Stable NEIL2 Transfectant**—Human gastric epithelial (AGS) cells were transfected with 1 µg each of NEIL2-FLAG or the empty vector (23). After 24 h, the cells were trypsinized and plated in fresh medium in the presence of zeocin to select clones carrying stably integrated plasmid DNA. The zeoocin sensitivity of those cells was determined to be ~200 µg/ml. The medium was replaced every other day with 250, 500, and 1000 µg/ml zeocin. After 3 weeks, individual zeoocin-resistant clones were transferred into 6-well plates.
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(0.25%) and nonfat dry milk. The blots were then probed with affinity-purified NEIL2 Ab and anti-rabbit secondary Ab and detected using an ECL kit (GE Healthcare).

Analysis of NEIL2 Activity—DNA strand cleavage at the site of the lesion due to the intrinsic AP lyase activity of NEIL2 (5' OHU-B11) or duplex (5'-OHU-G) or single-stranded (5'-OHU) DNA (15). These oligonucleotide substrates (2 pmol) were then incubated with NEIL2 (0.2 pmol) alone or plus different amounts of hnRNP-U or RPA (0.05, 0.1, and 0.2 pmol) in a buffer containing 40 mM Hepes-KOH, pH 7.5, 50 mM KCl, 1 mM MgCl₂, 5% glycerol, and 1 mg/ml BSA at 37 °C for 10 min with the 5'-OHU-containing oligonucleotide. The reactions were stopped with 70% formamide, 30 mM NaOH, and the product reactions were analyzed by denaturing gel electrophoresis (29).

Preparation of Single Lesion-containing Plasmid Substrate—Plasmid DNA (a gift from Philip Hanawalt) substrates containing 5'-OHU, in either the transcribed strand or the nontranscribed strand, were generated using pUC-TS (where TS represents transcribed strand) and pUC-NTS (where NTS represents nontranscribed strand) by following the protocol described previously with some modifications (30). Covalently closed circular (form I) plasmids containing a single lesion were generated by priming 5 μg of the plus strand of pUC-TS or pUC-NTS with a 20-fold molar excess of damage-containing oligonucleotide phosphorylated at the 5'-OHU site.

Plasmid DNA was confirmed by treating an aliquot with ethidium bromide. The plasmid contains an adenoviral late promoter; the lesion was introduced 167 bp downstream of the transcription start site. The presence of 5'-OHU in the promoter due to the intrinsic AP lyase activity of NEIL2 represents nontranscribed strand) by following the protocol described previously with some modifications (30). Covalently closed circular (form I) plasmids containing a single lesion were generated by priming 5 μg of the plus strand of pUC-TS or pUC-NTS with a 20-fold molar excess of damage-containing oligonucleotide phosphorylated at the 5'-end (5'-P-CCC-CGGGXACCGG-3', where X represents 5'-OHU) in a 300-μl reaction mixture containing 10 μl Tris-HCl (pH 7.9); 50 mM NaCl; 10 mM MgCl₂; 1 μM DTT; 60 μM each dATP, dCTP, dGTP, and dTTP; 1 mM ATP; 30 units of T4 DNA polymerase; and 5 units of T4 DNA ligase. Form I DNA was purified after electrophoresis in an agarose gel containing 0.25% gelatin and 10 mM ethidium bromide. The plasmid contains an adenoviral late promoter; the lesion was introduced 167 bp downstream of the transcription start site. The presence of 5'-OHU in the plasmid DNA was confirmed by treating an aliquot with E. coli Nth, which cleaved the damage-containing strand at the 5'-OHU site.

Transcription-coupled Base Excision Repair (TC-BER) with NEIL2-FLAG Immunocomplex—The immunocomplexes isolated from stable transfectants of vector control or NEIL2-FLAG cells were washed with a buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, protease inhibitor mixture) containing 10% glycerol. The bead-bound immunocomplexes were then used directly in repair reactions with the 5'-OHU-containing plasmid DNA (pUC-TS and -NTS) in the absence and presence of NTPs as indicated in the figure (Fig. 4B). The reaction buffer (50 μl) contained 25 mM HEPES-KOH, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 50 μM each dNTP, 2 μCi of [α-32P]dATP (the concentration of the corresponding cold dNTP was lowered to 5 μM unless otherwise specified). After incubation for 60 min at 30 °C, the plasmid DNA was phenol/chloroform-extracted, ethanol-precipitated, recovered, and digested with EcoRI/HindIII to separate a fragment spanning the repaired site for 5'-OHU and then resolved on a 6% denaturing polyacrylamide gel and analyzed with a PhosphorImager.

Reconstitution of Transcribed Strand-specific BER—Repair reactions were carried out with the plasmid DNA substrates and various combinations of recombinant NEIL2, PNK, Lig IIIα, Pol β, and hnRNP-U (the protein concentrations being optimized for maximum product formation) and 21 μl of purified RNAP II fraction containing TBP, TFIIIB, E/F/H fraction (ProteinOne, Bethesda, MD) in reaction mixtures containing 50 mM HEPES-KOH, pH 7.9, 70 mM KCl, 5 mM MgCl₂, 1 mM DTT, dNTP (50 μM each), 2 μCi of [α-32P]dATP or [α-32P]dATP (the concentration of the corresponding cold dNTP was lowered to 5 μM unless otherwise specified) with or without 250 μM NTPs. The reactions were carried out at 30 °C for 90 min in a 50-μl reaction mixture and then stopped with 2% SDS in 0.2 M Tris-HCl (pH 7.5) and 10 μg/ml proteinase K. The nucleic acids were precipitated with ethanol, digested with EcoRI/HindIII, and once again ethanol-precipitated before final resuspension in formamide dye and denaturation at 90 °C for 3 min. Products were resolved alongside a φX174 HindII DNA marker (Promega) on a 6% denaturing polyacrylamide gel in Tris borate-EDTA containing 8 M urea. The gels were dried, and the repaired products were analyzed with a PhosphorImager. S.E. bars for all of the experiments were calculated from at least three independent experiments using Microsoft Excel 7.0.

Chromatin Immunoprecipitation (ChIP) and Re-ChIP Assay—We have used NEIL2-FLAG stable (AGS) and human neuroblastoma (SK-N-BE2-(C)) cells for the ChIP/re-ChIP assay. SK-N-BE2-(C) cells were cultured in RPMI 1640 medium containing 10% FBS, 1% glutamine, and 1% non-essential amino acids. When the cells were 60–70% confluent, fresh medium containing 10 mm retinoic acid was added to the cells, which were then allowed to differentiate for 3 days (31). ChIP analysis was performed using a chromatin immunoprecipitation assay kit (Upstate Cell Signaling Solution, Millipore, Temecula, CA) per the manufacturer’s protocol, and the re-ChIP assays were performed as described (32). Briefly, the cells (~10⁸ cells) were treated with formaldehyde (1% final concentration) for 10 min for cross-linking and then washed twice with PBS and lysed in 200 μl of cell lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS) with a protease inhibitor mixture and sonicated to generate ~400-bp-long DNA fragments, and the supernatants were diluted with 20 mM Tris-HCl pH 8.0, 1.0 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.01% SDS, and protease inhibitors). The pre-cleared supernatant was then incubated overnight at 4 °C with antibody to FLAG, RNAP II (Santa Cruz Biotechnology, Inc.), hnRNP-U, or NEIL2, as indicated in Fig. 6. The immune complexes were precipitated with salmon sperm DNA/protein G-agarose, and the agarose beads were washed sequentially in a low salt wash buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 0.1% SDS), a high salt wash buffer (same as low salt wash buffer except containing 500 mM NaCl), LiCl wash buffer (20 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% Nonidet P-40, 0.5% deoxycholate, and protease inhibitors), and 20 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer. The immune complexes were extracted from the beads...
NEIL2 Associates with the RNAP II Complex—The strong activity of NEIL2 with oxidized base lesions in bubble DNA suggested its preferential role in the repair of oxidized bases during transcription. We hence tested for the association of NEIL2 with RNAP II. A C-terminally FLAG-tagged, enzymatically active NEIL2 or the empty FLAG-vector was stably expressed in human gastric epithelial AGS cells. Co-IP analysis with an anti-FLAG Ab revealed the presence of RNAP II in the NEIL2 immunocomplex (Fig. 1A, lane 3). The reverse IP using RNAP II Ab (N20, Santa Cruz Biotechnology, Inc.) showed the presence of NEIL2-FLAG in the RNAP II immunocomplex (Fig. 1B, lane 3). We have also generated two other human cell lines (HCT116 and HEK293) stably transfected with NEIL2-FLAG and vector control and confirmed NEIL2-RNAP II association in these cells as well (data not shown).

We then examined whether the endogenous NEIL2 and RNAP II associate in cells. The immunoprecipitation of endogenous NEIL2 from normal AGS cell extract using anti-NEIL2 Ab clearly showed the presence of RNAP II in the NEIL2 immunocomplex (Fig. 1C, lane 3). Furthermore, the reverse IP using RNAP II Ab (N20) showed the presence of NEIL2 in the RNAP II immunocomplex as well (Fig. 1D, lane 2), indicating stable association between NEIL2 and RNAP II in the cells.

hnRNP-U Interacts with NEIL2—To identify the accessory proteins in NEIL2-initiated repair, we screened for its interacting partners via large scale affinity pull-down and mass spectroscopy (MALDI-TOF-TOF) analysis. Two bands present in the NEIL2-FLAG complex (~120 and 100 kDa) subsequently identified as hnRNP-U and nucleolin (Fig. 2A, lane 1), were absent in the empty FLAG-vector complex (lane 2). The presence of hnRNP-U in the NEIL2-FLAG immunoprecipitate was confirmed by Western analysis of the immunocomplex (Fig. 2B, lane 3). We also confirmed the physical association of nucleolin with NEIL2; however, the functional significance of this interaction remains to be explored.

Next, His pull-down experiments using purified His-tagged hnRNP-U (Fig. 2C, lane 3) and far Western analysis with recombinant NEIL2 (28) showed a direct binary interaction in vitro between hnRNP-U and NEIL2 (Fig. 2D, lane 4).

hnRNP-U Stimulates NEIL2 Activity—We tested the functional implications of the interaction between hnRNP-U and NEIL2 by analyzing the strand incision activity of NEIL2 at a 5-OHU lesion site located in a 51-mer oligonucleotide substrate present as single-stranded DNA, duplex DNA, or a partial duplex formed with the lesion in an 11-nt bubble (supplemental Fig. S1) (15). NEIL2 activity was stimulated 5–6-fold by hnRNP-U in a dose-dependent manner (Fig. 3A, lanes 4–6; compare with lane 3) with single-stranded and bubble DNA and 3–4-fold with the duplex DNA. As expected, hnRNP-U itself had no DNA glycosylase activity (lane 2). To examine further the specificity of stimulation by hnRNP-U, we tested the effect of RPA, the major single-stranded DNA-binding protein in mammalian cells, on NEIL2 activity (Fig. 3B, lanes 7–9). Instead of stimulating it, RPA inhibited the activity of NEIL2 with bubble and single-stranded DNA, presumably by binding to the DNA and thus preventing access of NEIL2 to the damage. Furthermore, the addition of neutralizing hnRNP-U Ab to the reaction mix containing both NEIL2 and hnRNP-U abolished stimulation by hnRNP-U (Fig. 3B, lane 5); however, the Ab alone had no effect on NEIL2 activity (lane 3). Restoration of stimulation by the addition of excess hnRNP-U confirmed the specificity of the hnRNP-U-NEIL2 interaction (lane 6).

The NEIL2 Immunocomplex Is Proficient in 5-OHU Repair in the Transcribed Strand—After identifying hnRNP-U and RNAP II in the NEIL2 immunocomplex, we tested for the presence of other repair proteins in the complex and detected Pol β, PNK, and Lig IIIα (Fig. 4A), as we had reported earlier.
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A. Coomassie stain  
B. NEIL2-FLAG IP

![Images of gel stains and immunoprecipitates]

We next tested the ability of this complex (isolated from AGS cells) to repair a plasmid DNA containing the base lesion placed in the transcribed (pUC-TS-5-OHU) versus the non-transcribed strand (pUC-NTS-5-OHU; Fig. 4B). It was recently shown that the oxidative lesions 5-OHU, thymine glycol, and 8-oxoguanine all are bypassed during transcription in a reconstituted system, albeit to varying extents. When bypassed by RNAP II, the 5-OHU lesion is the most likely among these to produce a mutant transcript (33). We thus used 5-OHU-containing plasmid DNA in the repair assay. Both pUC-TS and NTS-5-OHU plasmid DNAs were incubated with equal amounts of NEIL2-FLAG or the FLAG-vector immunoprecipitates in the presence or absence of NTPs (Fig. 4C). To monitor 5-OHU repair, we analyzed the incorporation of $[\alpha-32P]dTMP$ into the plasmid containing a 5-OHU residue in the transcribed strand (TS) opposite A in the non-transcribed strand (NTS). Although negligible radioactivity was incorporated in the plasmid DNA with the lesion in the TS using an immunocomplex isolated from the empty vector-expressing cells (Fig. 4C, lanes 6 and 7), the NEIL2-FLAG immunoprecipitate catalyzed significant incorporation of $[\alpha-32P]dTMP$ in the plasmid, indicating repair, especially when NTPs were added in the incubation mixture to allow transcription (lane 2 versus lane 3). On the other hand, repair of the plasmid with the lesion in the NTS was significantly less even in the presence of NTPs (lane 4 versus lane 5). Furthermore, $\alpha$-amanitin strongly inhibited (6–7-fold) dTMP incorporation in the TS strand (Fig. 4D, lane 3 versus lane 2), suggesting that ongoing transcription is required for efficient repair. Importantly, NEIL2-FLAG immunoprecipitate is competent in generating the full-length transcript (365 nt) from both pUC-TS-5-OHU and pUC-NTS-5-OHU (Fig. 4E, lanes 3 and 4). To investigate the effect of hnRNP-U on NEIL2-initiated repair, we preincubated NEIL2-FLAG immunoprecipitate with a neutralizing hnRNP-U Ab and observed a significant reduction in the extent of repair (~5-fold; Fig. 4F, lane 3 versus lane 2). This inhibitory effect was partially reversed by the addition of purified hnRNP-U to the NEIL2-FLAG immunoprecipitate (lane 1), confirming the critical role of hnRNP-U in the repair process. Taken together, these results indicated NEIL2-initiated preferential repair of the transcribed strand that also required active transcription.

Reconstituted System for Preferential Repair of the Transcribed Strand—Given our finding that the NEIL2-FLAG immunocomplex is proficient in repair of 5-OHU from the transcribed strand and our partial characterization of NEIL2-associated proteins in the complex (Fig. 4A), we asked whether transcription-dependent repair of oxidized bases could be demonstrated in a reconstituted system using purified proteins and a plasmid DNA containing 5-OHU at a specific site in the transcribed strand (pUC-TS-5-OHU; Fig. 4B). The plasmid DNA substrate was incubated with optimized reaction mixture (365 nt) from both pUC-TS-5-OHU and pUC-NTS-5-OHU (Fig. 4C). On the other hand, repair of the plasmid with the lesion in the NTS was significantly less even in the presence of NTPs (lane 4 versus lane 5). Furthermore, $\alpha$-amanitin strongly inhibited (6–7-fold) dTMP incorporation in the TS strand (Fig. 4D, lane 3 versus lane 2), suggesting that ongoing transcription is required for efficient repair. Importantly, NEIL2-FLAG immunoprecipitate is competent in generating the full-length transcript (365 nt) from both pUC-TS-5-OHU and pUC-NTS-5-OHU (Fig. 4E, lanes 3 and 4). To investigate the effect of hnRNP-U on NEIL2-initiated repair, we preincubated NEIL2-FLAG immunoprecipitate with a neutralizing hnRNP-U Ab and observed a significant reduction in the extent of repair (~5-fold; Fig. 4F, lane 3 versus lane 2). This inhibitory effect was partially reversed by the addition of purified hnRNP-U to the NEIL2-FLAG immunoprecipitate (lane 1), confirming the critical role of hnRNP-U in the repair process. Taken together, these results indicated NEIL2-initiated preferential repair of the transcribed strand that also required active transcription.
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Transcribed BER process. Taken together, these results strongly suggest that NEIL2-initiated repair of 5-OHU in the transcribed strand is transcription-dependent.

NEIL2 Associates with RNAP II and hnRNP-U on Transcribed Genes—Our results support a model in which NEIL2, RNAP II, and hnRNP-U associate to form a com-

FIGURE 3. Stimulation of NEIL2 activity by hnRNP-U. A, bubble (5-OHU-B11; top), duplex (5-OHU-G; middle), or single-stranded (5-OHU; bottom) oligonucleotide (2 pmol) was incubated at 37 °C for 10 min with hnRNP-U (0.2 pmol; lane 2), NEIL2 (0.2 pmol; lane 3), or RPA alone (0.2 pmol; lane 10) or with NEIL2 (0.2 pmol) with increasing amounts of hnRNP-U (0.05, 0.1, and 0.2 pmol; lanes 4–6) or RPA (0.05, 0.1, and 0.2 pmol; lanes 7–9). S, substrate; P, product. The histograms show quantitation of the cleaved products, setting the activity of NEIL2 alone (lane 3) to 1. Values for these histograms and those in all other figures represent the mean from at least three independent experiments. B, specificity of the stimulatory activity of hnRNP-U. Two pmol of substrate DNA (5-OHU-B11) was incubated at 37 °C for 10 min with NEIL2 (0.2 pmol) in the absence (lane 4) or the presence (lane 5) of anti-hnRNP-U Ab or anti-hnRNP-U Ab plus excess hnRNP-U protein (+ +, lane 6). Lane 1, no protein; lanes 2 and 3, NEIL2 alone and NEIL2 plus anti-hnRNP-U Ab, respectively. The histograms show quantitation of the cleaved product relative to NEIL2 alone (lane 2). Error bars, S.E.
FIGURE 4. TC-BER using NEIL2-FLAG immunocomplex. A, partial characterization of the NEIL2-FLAG immunocomplex by Western blot analysis. Extracts from AGS cells stably expressing NEIL2-FLAG (lane 3) or vector alone (lane 4) were immunoprecipitated with anti-FLAG Ab and tested for the presence of NEIL2-associated proteins with Abs to the proteins shown on the right. B, scheme for analysis of transcription-coupled BER using plasmid DNA containing a single 5-OHU in the transcribed or non-transcribed strand (pUC-TS-5-OHU or -NTS-5-OHU, respectively). The plasmids contain an adenoviral late promoter (gray box); the lesion (*) was introduced 167 bp downstream of the transcription start site (shown with an arrowhead) (30). C, repair of pUC-TS-5-OHU (lanes 2 and 3 and lanes 6 and 7) or pUC-NTS-5-OHU (lanes 4 and 5) plasmid DNAs using NEIL2-FLAG (lanes 2–5) or FLAG-vector immunoprecipitate (lanes 6 and 7), with (+) or without (−) 250 μM NTPs. Lane 1, 5′-end-labeled EcoRI/HindIII fragment from pUC-TS plasmid DNA as a size marker. Other details are provided under “Experimental Procedures.” The repaired plasmid was cleaved with EcoRI/HindIII and analyzed in a 6% TBE-urea gel. Complete repair is indicated by radioactive dTMP incorporation in the plasmid fragment spanning the 5-OHU lesion site. The histograms show quantitation of the repaired product relative to that in the TS without NTPs (lane 3). D, effect of α-amanitin on NEIL2-initiated BER. The repair assays were carried out using NEIL2-FLAG immunoprecipitate in the presence (lane 2) or absence (lanes 1 and 3) of α-amanitin and in the absence (lane 1) or presence (lanes 2 and 3) of NTPs, respectively. Quantitation of the repaired product is shown in histograms at the bottom; repair activity without α-amanitin (lane 3) and with NTPs was set to 1. E, transcript analysis. The NEIL2-FLAG immunocomplex was incubated for 1 h at 30 °C with 200 ng of pUC-TS-5-OHU (TS, lane 3) or pUC-NTS-5-OHU (NTS, lane 4) plasmid DNA (30) in a 50-μl reaction buffer (25 mM HEPES-KOH, pH 7.9, 50 mM KCl, 5 mM MgCl₂, 0.5 mM DTT) containing 20 units of RNase inhibitor and a 250 μM concentration each of GTP, CTP, and ATP; 10 μM UTP; and 5 μCi of [α-32P]UTP. The reaction mixture was phenol/chloroform-extracted, ethanol-precipitated, and analyzed by denaturing gel electrophoresis. Lanes 1 and 2, marker DNAs 5′-labeled with [α-32P]ATP; F, the specificity of hnRNP-U in NEIL2-initiated BER was tested using the NEIL2-FLAG immunocomplex either in the absence (lane 3) or presence (lanes 1 and 2) of anti-hnRNP-U Ab. The effect of the anti-hnRNP-U Ab could be partially reversed by the addition of purified hnRNP-U protein (0.2 pmol; lane 1). The histograms show quantitation of the repaired product relative to the repair activity of NEIL2-FLAG immunoprecipitate (lane 3). Lane 4, marker. Error bars, S.E.
Reconstitution of TC-BER

| Control DNA | (TS) | $\alpha_32^{32P}$ dATP |
|-------------|------|------------------------|
| $\alpha$-amanitin | - | - |
| TFs | + | - |
| RNAP II | + | + |
| PNK, Pol$\beta$, LigIII$\alpha$ | + | + |
| NEIL2 | + | - |
| hnRNP-U | + | - |
| NTPs | + | + |

| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------|---|---|---|---|---|---|---|---|---|
| Value | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |

**FIGURE 5. In vitro reconstitution of TC-BER.** For radioactive incorporation into pUC-TS-5-CHU (with $[\alpha-32P]$dTMP (lanes 1–7) or $[\alpha-32P]$dAMP (lane 8)), 10 pmol of substrate was incubated with 0.5 pmol of NEIL2 (lanes 1 and 3–8), 25 fmol of PNK, 0.25 pmol each of Pol $\beta$ and Lig III$\alpha$, and an RNAP II fraction containing TBP, TFIIH, and E/F/H (Protein One, except in lane 5 without the transcription factors) in the presence (+) or absence (−) of NTPs, as described under “Experimental Procedures.” Lane 1, undamaged DNA; lane 9, end-labeled $\phi$X174 HindIII DNA marker (Promega). Complete repair is indicated by the $[\alpha-32P]$dTMP incorporation. The repaired plasmid was linearized with EcoRI/HindIII and analyzed in a 6% TBE-urea gel; the gel was dried and the reaction products were analyzed and quantitated with a PhosphorImager. **Bottom,** histograms indicating the fold-increase in repair activity; activity without NTPs (lane 3) was set to 1. Error bars, S.E.

complex to preferentially repair the transcribing genes. To confirm association of these proteins on the transcribed genes, we carried out ChIP followed by re-ChIP from extracts of two different cell lines (NEIL2-FLAG expressing AGS and neuroblastoma SK-N-BE2-(C)) to resolve whether all three proteins were enriched on similar regions of the transcribing genes. In the ChIP/re-ChIP assay, we divided soluble chromatin fractions derived from cross-linked cells into two aliquots. We immunoprecipitated an aliquot with anti-FLAG Ab (Fig. 6A, AGS) or anti-NEIL2 Ab (Fig. 6A, SK-N-BE2-(C)), washed the immunoprecipitate, released the bound immune DNA complexes by elution, and prepared them for a second immunopull-down (re-ChIP) with anti-RNAP II Ab (lane 1) or IgG (lane 2). We selected several transcribed and non-transcribed genes in the gastric epithelial (AGS) cells and amplified the precipitated DNA by PCR using the corresponding gene-specific primers (supplemental Fig. S2). Fig. 6A shows strong association of NEIL2 and RNAP II with the transcribed genes but not with non-transcribed genes. Re-ChIP experiments using reciprocal Abs showed similar results (lane 3). This binding was specific, because re-ChIP experiments using control IgG (Fig. 6A, lanes 2 and 4) or involving NEIL2 and APE1 (AP endonuclease 1) (Fig. 6B) did not amplify those genes. We had shown previously that APE1 is absent from the NEIL2 immunocomplex, and hence our re-ChIP experiments are consistent with our previous findings (23).

To validate the association between NEIL2 and hnRNP-U, we have conducted similar ChIP/re-ChIP experiments and observed amplification of the transcribed (Fig. 6A, lanes 6 and 8) but not the non-transcribed genes. We selected neuron-specific genes that are not transcribed in AGS cells. To validate our results, we further conducted re-ChIP experiments in SK-N-BE2-(C) cells and found that NEIL2 does associate with RNAP II and hnRNP-U on the transcribed genes but not with MYOD, a repressed gene in SK-N-BE2-(C) cells. All of these findings are consistent with our biochemical characterization of an association of NEIL2 with RNAP II and hnRNP-U, which demonstrates the presence of all three proteins on transcribed genes and implicates their involvement in the repair of transcribing genes.

**NEIL2 Preferentially Repairs the Transcribed Genes**—To provide direct evidence for the preferential role of NEIL2 in repairing actively transcribing genes, we have generated stable NEIL2 and control shRNA-expressing (shRNA-N2 and shRNA-C) cell lines. The shRNA-N2-expressing cells showed an ~80% reduction in NEIL2 level (Fig. 7A, lane 2) compared with shRNA-C-expressing cells (lane 1). The cellular DNA was isolated from both cells, and the relative levels of base damage in the actively transcribed (β-actin and MYOD) versus non-transcribed genes (MASH and NEUROD), were compared in NEIL2-depleted versus control cells using quantitative real-time PCR (primer sequences in supplemental Fig. S2). The DNA samples were treated with E. coli Fpg/Nei before PCR to excise oxidized bases and generate single-strand breaks after excision of the damaged bases, thereby preventing PCR amplification (34). Thus, the fraction of damaged target genes can be assessed by the increase in the cycle numbers. There was a marked increase in the Ct values for the transcribed genes in NEIL2-depleted compared with control cells, reflecting a higher DNA damage level in NEIL2-deficient cells (Fig. 7B). In contrast, the damage in the non-transcribed genes was not significantly different in NEIL2-downregulated versus control cells (Fig. 7B). These data thus confirm the critical role of NEIL2 in repairing oxidized bases in the transcribed genes.

**DISCUSSION**

Preferential removal of lesions from the transcribed genes, formally called transcription-coupled repair, is essential for normal cellular functions and survival. Transcription-coupled repair was discovered as a distinct subpathway of NER (16, 17). All cells, regardless of whether cycling or postmitotic, must carry out transcription to maintain cellular functions and survival. Repair of transcribed sequences is thus more critical for cellular survival than is repair of the non-transcribed sequences, which
comprise the bulk of the genome. Transcribed gene-specific repair of base damage is particularly important in terminally differentiated, non-dividing cells, which represent >90% of cells in adult mammals. In such cells, where mutation fixation by replication is not a concern, only repair of the functional genes is necessary to prevent synthesis of mutant RNAs and proteins (35). Thus, preferential repair of oxidized lesions in active sequences in mammalian genomes should be at least as important as that of the bulky adducts that are repaired via NER and perhaps more so because the former are the predominant lesions induced by endogenous reactive oxygen species. However, the occurrence of TC-BER of oxidized bases and its potential mechanism were heretofore largely unknown. It is reasonable to postulate that initiation of TC-BER of oxidized bases might differ from TC-NER of bulky adducts that completely block transcription. There is strong evidence that stalled RNAP II at bulky adducts activates the TC-NER pathway (36). However, the extent to which oxidized bases block transcription is unclear, with differing results depending not only on the nature of the lesion but also on the transcription system used (30, 33, 37, 38) and possibly on the sequence context of the lesion.

A transcription-blocking lesion will be located within the transcription bubble formed by the elongating RNAP II, in which DNA strands are transiently separated (39, 40). For BER, the lesion must be removed from the single-stranded region of the bubble. This is in contrast to NER, in which dual incision occurs at the junctions between single-stranded and double-stranded DNA (39, 40). Our previous

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**FIGURE 6. ChIP/re-ChIP assay.** After sonication and immunoprecipitation of cross-linked chromatin with the first Ab (1st IP), the bound fraction was eluted, divided into two aliquots, and subjected to a second round of immunoprecipitation (2nd IP) with IgG (as control) or the specific Ab. A, ChIP/re-ChIP assay involving NEIL2-RNAP II or NEIL2-hnRNP-U. PCR amplifications were performed using the primers flanking the coding regions of actively transcribed or repressed genes in AGS and SK-N-BE2-(C) cells. Note that lanes 1 and 3 and lanes 6 and 8 each show corresponding assay pairs in the reciprocal order. B, ChIP/re-ChIP assay involving NEIL2 and APE1. Cross-linked chromatin was immunoprecipitated from NEIL2-FLAG-expressing AGS cell extract with anti-FLAG (lane 2) or anti-APE1 (lane 3) Ab, and the NEIL2-bound fraction was then subjected to a second round of immunoprecipitation with IgG (lane 4) or anti-APE1 (lane 5) Ab. PCR amplifications were performed using primers flanking the coding regions of β-actin, transcribed in all cell types. Other details are provided under “Experimental Procedures.”
observations of preferential lesion removal from bubble DNA by NEIL2 and of cell cycle-independent expression of NEIL2 suggest NEIL2 as the candidate DNA glycosylase for initiation of TC-BER. Here we show that NEIL2 constitutively associates with the elongating form of RNAP II in the cell (Fig. 6). To our knowledge, our studies here showed for the first time the association of a DNA glycosylase with the elongating RNAP II. These findings further implicate NEIL2 in TC-BER.

In an effort to unravel the in vivo functions of NEIL2, we screened for additional interacting partners and identified hnRNP-U, among others (Fig. 2A). hnRNP-U, a multifunctional nucleoplasmic protein, binds pre-mRNA in vivo and RNA and single-stranded DNA in vitro (41). Interestingly, hnRNP-U was shown to cooperate in enhancing RNAP II-mediated transcription (42) and also interacts with components of the transcription machinery, including TFIIH (43) and the transcriptional coactivator p300. Association of p300 and hnRNP-U could promote histone acetylation and chromatin unfolding in active chromatin regions before transcription (44). It is noteworthy that p300 has been identified as a component of transcription-coupled repair complexes in the cell (45).

Recently, hnRNP-U was shown to be involved in double strand break repair (46). Here we report its role in BER as well. Interaction with hnRNP-U stimulates removal of a 5-OHU lesion from bubble DNA by NEIL2. Such a key role of hnRNP-U in BER of oxidative DNA damage, probably in association with transcription, was quite unexpected. These results, together with the fact that hnRNP-U, also identified independently as scaffold attachment factor A, is a major component of the nuclear matrix (47), suggest a critical dynamic role of the nuclear architecture in the repair of oxidized bases during transcription (48).

Using the NEIL2-FLAG immunocomplex, we have developed an assay for preferential repair of 5-OHU in the transcribed strand. Notably, the NEIL2-FLAG immunoprecipitate is competent to generate full-length transcript. This is a remarkable observation, considering the complexities of promoter-initiated transcription; these results provide further support for transcription-dependent repair-proficient complex formation (Fig. 4). We then developed a reconstituted in vitro system that allows both transcription and BER on a plasmid DNA substrate (Fig. 5). Notably, NEIL2-initiated repair of the transcribed strand also requires hnRNP-U. In cultured cells, however, for technical reasons, we were able to demonstrate transcription-adenised strand-specific repair. Of further importance, we showed in ChIP/reChIP experiments that NEIL2, RNAP II, and hnRNP-U are enriched on transcriptionally active but not on repressed genes. We thus hypothesize that hnRNP-U is the major molecular link between transcription and repair. Finally, we have shown that NEIL2-down-regulated cells accumulate additional damage in the transcribed but not the repressed genes. Taken together, our observations clearly demonstrate NEIL2-mediated transcribed gene-specific BER of oxidative DNA damage.

It is important to emphasize that our reconstituted system involves only the proteins that were partially characterized in the NEIL2 immunocomplex (Fig. 4A). Many other proteins, including CSB, XPG, and mismatch repair complexes as well as chromatin-remodeling proteins, may also be involved in transcription-coupled repair of oxidized bases in the cell (39, 45, 49, 50). Transcription-coupled repair and its regulation are obviously complex and probably also involve other unidentified proteins. For example, a recently characterized co-crystall structure of backtracked RNAP II and TFIIIS suggests a critical role of TFIIIS in rescuing the complex from the arrested state (51), so TFIIIS could also be involved in TC-BER. Furthermore, transcription elongation by RNAP II is an exquisitely complex process, especially in the context of chromatin. Several elongators and other factors have been proposed to assist phosphorylated RNAP II during elongation (52). These issues will be explored in future studies.

Various oxidized DNA base lesions, including 5-OHU, were recently shown to be bypassed during RNAP II-mediated transcription to variable extents in vitro (33). These studies suggest the involvement of multiple factors in allowing transcription to bypass oxidized bases, with the probability of insertion of incorrect nucleotides in the transcript being dependent on the nature of the lesion. Our studies underscore the likely presence of a cellular mechanism to prevent such transcriptional mutagenesis due to unrepaired base lesions through their preferential repair by NEIL2 during transcription. Our results, together with the recent findings on transcriptional bypass, raise the possibility that hnRNP-U and other factors (including CSB, XPG, elongin, and TFIIIS) may be recruited to a transcription repair complex in vitro to facilitate transcription of templates containing oxidative DNA damage in mammalian genomes. Despite uncertainty about the mechanistic details, the important feature of this process is that NEIL2 plays a critical role in maintaining genomic integrity via preferential repair of oxidatively damaged bases in the transcribed genes. The role of hnRNP-U and possibly other factors in bestowing specificity for repairing the transcribing genes obviously warrants further investigation.
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