Coordination between Polymerase β and FEN1 Can Modulate CAG Repeat Expansion*

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Trinucleotide repeat (TNR) expansion has been identified as a cause of more than 40 human neurodegenerative diseases, including Huntington disease (CAG/CTG), myotonic dystrophy type 1 (CTG/CAG), Friedreich ataxia (GAA/TTC), Fragile X syndrome (CGG/CCG), and many others (1, 2). The instability of CAG repeats also is associated with human cancer (3, 4). TNR expansion that occurs in a protein-encoding region of the genome may result in alteration of the encoded protein. An example of this was found in the case of CAG repeat expansion leading to a polyglutamine segment in an encoded protein. This results in aggregation of the altered protein, a condition associated with cell death and neurodegeneration (5). Repeat expansion in non-encoding regions also can result in structured RNA transcripts of specific genes that are targeted by a Dicer-mediated RNA degradation mechanism (6, 7), thereby reducing mRNA levels for the target genes.

One component of the molecular basis underlying TNR expansion is considered to be the formation of non-B-form or noncanonical DNA structures (8–10). These structures include intrastand base-paired hairpins (CNG/CNG), tetraplexes (CGG/CCG), triplexes (GAA/TTC) (12), and so-called “sticky” DNA (GAA/TTC) (9, 10). The noncanonical DNA structures can defeat DNA repair mechanisms by blocking repair enzymes, and persistence of these structures ultimately leads to their stable integration into the genome resulting in TNR expansion or contraction (13). In contrast, DNA metabolic pathways such as replication (14–16), repair (17, 18), and recombination (19, 20) can modulate TNR stability by eliminating DNA noncanonical structures. Interestingly, disease-associated TNR expansions result in pathology in brain and skeletal muscle where many of the cells are postmitotic (21–24). As an example, somatic CAG repeat expansion is observed in terminally differentiated striatal neurons and exhibits an aging dependence (25, 26). This cannot be explained by a DNA replication-based mechanism (27). It is also unlikely that the expansion results from DNA recombination, as neuronal CAG repeats typically exhibit small expansions limited to ~1–20 repeat units (25, 26), whereas a recombination-based mechanism is expected to result in larger repeat changes (19).

Although mismatch repair proteins have been shown to be essential for CAG repeat expansion (17, 28), this repair pathway does not appear to account for the aging-dependence of the repeat expansion. Because aging is considered to involve increased oxidative stress (29, 30), it is reasonable to suggest that the age-dependent neuronal CAG repeat expansion involves oxidative DNA damage (27, 31). Indeed, a linkage between the repeat expansion in brain and oxidative DNA damage was established in a recent study (25).

Endogenous and exogenous oxidative stress can cause cellular accumulation of reactive oxygen species and other types of oxidative free radicals (32). These reactive species can result in oxidized DNA bases, such as 8-oxoG, apurinic/apyrimidinic (AP) sites, and single-stranded DNA (ssDNA) breaks (33). These DNA lesions are repaired by pol β-dependent BER. The DNA glycosylase OGG1 removes 8-oxoG, leaving either an intact AP site or a single nucleotide gap with a 3’-O blocking group and 5’-phosphate groups at the margins (34). In the former case, AP endonuclease 1 (APE1) incises the intact AP site, resulting in a single nucleotide, gapped DNA with 3’-OH and 5’-phosphate groups (35). A summary of the roles of AP endonuclease 1 (APE1) and DNA flap endonuclease 1 (FEN1) in DNA repair is shown in Table 1.
5'-dRP groups at the margins (35). In the latter case, APE1 and/or a polynucleotide kinase removes the 3'-O blocking group. Subsequently, pol β fills the single nucleotide gap and removes the 5'-dRP group, if present (36–40), creating a nicked DNA that is a substrate for ligase. The DNA ligase IIIα-x-ray cross-complementing group I (XRCC1) complex or DNA ligase I seals the nick to accomplish the final step in mammalian single nucleotide BER (SN-BER). Alternatively, in a scenario in which oxidative stress results in an oxidized 5'-sugar phosphate group that is refractory to pol β dRP lyase activity, the damage may be repaired by the long-patch BER (LP-BER) subpathway (36, 37, 41–43). In one version of this subpathway, pol β fills the single nucleotide gap, resulting in a nicked DNA with the oxidized 5'-sugar phosphate, creating a single nucleotide gap for pol β to fill. This sequential coordination between pol β and FEN1 has been referred to as the “hit-and-run” mechanism for LP-BER (44). Steps in BER enzymology that may lead to TNR expansion could include generation of ssDNA breaks, DNA slippage to form hairpin structures, multiligase gap-filling DNA synthesis, and ligation to incorporate intrastrand hairpins into repaired strands. A functional deficiency or imbalance in BER enzymes could cause accumulation of intermediates such as ssDNA breaks or hairpin structures that hasten TNR expansion.

The discovery of a requirement for OGG1 in CAG repeat expansion in the Huntington disease mouse model was consistent with the idea that OGG1 initiates repair leading to ssDNA breaks, which subsequently increases the chance of repeat expansion (25). Yet, this requirement for OGG1 raised many questions. For example, can expansion occur during cell extract-based BER of the 8-oxoG lesion in the context of CAG repeats? If so, which BER enzymes and cofactors are required in the expansion, and is the chance of expansion during BER modulated by the levels of the enzymes and cofactors? In the present study, we observed CAG repeat expansion during cell extract-based BER as well as reconstructed BER of 8-oxoG imbedded in the repeat sequence and then explored the roles of APE1, pol β, FEN1, and HMGB1 in modulating this CAG repeat instability. The results point to the conclusion that disruption of the usual coordination between pol β and FEN1 leads to CAG repeat expansion.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA oligonucleotides containing 8-oxoG were from Operon Biotechnologies Inc. (Huntsville, AL). All other oligonucleotides were from Integrated DNA Technologies Inc. (Coralville, IA). The radiolabeling [γ-32P]ATP (7000 mCi/mmole) was from MP Biomedicals (Irvine, CA). Micro Bio-Spin 6 chromatography columns were from Bio-Rad. Deoxynucleoside 5'-triphosphates were from Roche Diagnostics. Oligokine was from USB Corp. (Cleveland, OH). *Escherichia coli* T5 exonuclease was from Epicenter (Madison, WI). DNase I was from Invitrogen, and most other reagents were from Sigma-Aldrich. The recombinant human BER proteins used (OGG1, APE1, pol β, FEN1, and HMGB1) were purified as described previously (25, 45–48).

**Oligonucleotide Substrates**—DNA oligonucleotide substrates containing 8-oxoG were designed and synthesized as described previously (25). Substrates containing a tetrahydrofuran (THF), an abasic site analog and natural abasic site, were designed to mimic the product from monofunctional OGG1 removal of 8-oxoG. The guanine in the first CAG counted from the 5'-end of a substrate was substituted with the THF residue or a deoxyuridine. Substrates with a natural abasic site were created by incubating oligonucleotides containing a deoxyuridine residue with *E. coli* uracil-DNA glycosylase (50 nm) at 37 °C for 30 min. Substrates containing (CAG)20 or (CAG)25 with 8-oxoG were utilized in the BER reconstitution experiments. Substrates containing 5'-THF-(CAG)12 flap/hairpin or 5'-THF-(CAG)20 hairpin were designed to create an intermediate resulting from pol β multinucleotide gap-filling synthesis. They were constructed by annealing an upstream primer containing (CAG)12 or (CAG)20 and a downstream oligonucleotide containing 5'-THF-(CAG)12 flap/hairpin, or the 5'-THF-(CAG)20 hairpin, to a template strand. BER substrates were prepared by annealing an oligonucleotide strand with base damage to its template strand at a molar ratio of 1:1.5 (template) or by annealing upstream and downstream oligonucleotides to their template strand at a molar ratio of 1:1:1.5 (template). All substrates were radiolabeled at the 5'-end of the damage-containing strand or the downstream strand DNA as specified in Figs. 1–7. The sequences and descriptions of the oligonucleotides used are shown in Table 1. DNA markers were identical to the CAG repeat-containing substrates, except they were damage-free; they corresponded to 100-nucleotide (CAG)20, 115-nucleotide (CAG)25, and 130-nucleotide (CAG)30. The markers were synthesized and PAGE-purified by Integrated DNA Technologies. Additional DNA markers of 22, 25, 28, 31, 34, 37, and 40 nt used in Fig. 2 corresponded to the APE1 cleavage product and six pol β insertion intermediates after addition of dAMP. These markers were synthesized and PAGE-purified by Integrated DNA Technologies. The markers were 5’-radiolabeled and mixed in equal molar ratio.

**Protein Purification**—Human DNA ligase I (LIG I) expression vector, pET15b-N terminal Hisα-tagged DNA ligase I (49) was kindly provided by Dr. Jeffrey J. Hayes, Department of Biochemistry and Biophysics, University of Rochester Medical Center. The vector was introduced into *E. coli* BL21 AI strain (Invitrogen) by transformation. First, 500 ml of LB medium was inoculated with a freshly transformed single colony and agitated overnight at 37 °C. Then, 100 ml of this preculture was inoculated into five batches of LB medium (1 liter each), and bacteria were cultured at 37 °C with shaking until the cultures reached an absorbance of 0.5–0.8 at 595 nm. Then, LIG I expression of each culture was induced by addition of isopropyl-β-D-galactopyranoside to 1 mM and shaking for 24 h at 18 °C. Cells were harvested by centrifugation, resuspended in 150 ml of Buffer A (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.1% Nonident P-40, 0.1 μM 4-(2-aminoethyl)benzenesulfonl fluoride hydrochloride, 1 mM benzamidine, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin), and lysed by sonication. Cell lysates were subjected to centrifugation at 40,000 rpm for 40 min at 4 °C to separate soluble proteins from cell debris.
The purification of LIG I was accomplished using a two-column scheme. The supernatant fraction from the 1-liter culture described above was initially subjected to P11 phosphocellulose chromatography. A 60-ml bed volume P11 phosphocellulose column (Whatman-GE Healthcare) was equilibrated with 10 column volumes of Buffer A. The supernatant fraction was loaded onto the column and washed with 5 column volumes of Buffer A. The bound protein was eluted with a 10-column volume linear gradient of 50–1000 mM NaCl in Buffer A. Fractions were analyzed by SDS-PAGE and staining with Coomassie Blue. The protein peak fraction of LIG I eluted at ~500 mM NaCl. The LIG I-containing protein fractions were pooled, dialyzed into Buffer A containing 10 mM imidazole, and loaded onto a 1.5-ml nickel-nitritotriacetic acid column. The column was washed with 5 column volumes of a washing buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 7 mM 2-mercaptoethanol, 500 mM NaCl, 10 mM imidazole, 0.1 μM benzzenesulfonyl fluoride hydrochloride, 1 mM benzamidine, 1 μg/ml pepstatin A, and 1 μg/ml leupeptin). LIG I was eluted with a 10-column volume linear gradient of 15–600 mM imidazole in the same buffer. The peak fraction of LIG I eluted at ~300 mM imidazole. The protein fractions were pooled, concentrated with Amicon Ultra-15 (Amicon-Millipore, Billerica, MA), and stored in storage buffer containing 25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol. The purity of the final preparation was assessed by SDS-PAGE, where the sample was judged to be >99% pure, with loading of 20 μg of protein onto the gel. The activity of purified LIG I was determined by the ability of the enzyme to ligate radiolabeled nicked DNA in the presence of ATP. No contamination by bacterial DNA polymerases and nucleases was detected in routine assays for these enzymes. Purified LIG I was frozen in aliquots at −80 °C until use.

In vitro BER in Mouse Embryonic Fibroblast Cell Extracts—pol β null (−/−), wild-type (pol β+/+), and “pol β overexpression” mouse embryonic fibroblast cell lines were grown to near confluence. Cells were washed twice with phosphate-buffered saline and harvested with a cell scraper. Cell extracts, made as described previously (50), were dialyzed into buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mg/ml BSA, and 0.01% Nonidet P-40. In vitro BER of 8-oxoG-containing DNA in cell extracts was determined by incubation with 10 nm (CAG)20 repeat-containing substrate or a control random sequence-containing substrate, each with an 8-oxoG lesion, as indicated. The 20-μl reaction mixture contained 60 μg of cell extract, 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mg/ml BSA, 5 mM MgCl2, 0.01% Nonidet P-40, 4 mM ATP, and 50 μM dNTPs. Complementation experiments with pol β and HMGB1 were conducted by adding increasing concentrations of purified pol β or HMGB1 to pol β null and HMGB1 null cell extract (60 μg), respectively. The DNA substrates were preincubated with purified OGG1 (1 μM) and 50 nm APE1 at 37 °C for 30 min to generate ssDNA break BER intermediates. The reaction mixture was assembled on ice and then incubated at 37 °C for 30 min. The BER reaction was terminated by transfer to 95 °C for 5 min. The reaction mixture was subsequently subjected to protease K digestion at 55 °C for 30 min. DNA was precipitated and dissolved into buffer containing 95% formamide and 2 mM EDTA. DNA substrates and products were separated by 12–15% urea-denaturing polyacrylamide gel electrophoresis and were detected using a PhosphorImager (GE Healthcare). Expansion products were cut from gel and isolated by elution at 60 °C for 1 h with elution buffer (pH 7) containing 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, and 0.1% SDS. DNA products were precipitated, dissolved in water, and stored at −20 °C for subsequent size analysis. Substrates were 32P-radiolabeled at the 5′-end of the DNA strand containing 8-oxoG.

In Vitro BER Reconstitution with Purified Enzymes—BER of ssDNA breaks was reconstituted with purified BER enzymes, OGG1, APE1, pol β, FEN1, LIG I, or T4 DNA ligase (NEB, Ipswich, MA), and CAG repeat-containing substrates or random DNA sequence substrate with 8-oxoG in one strand. The 20-μl reaction mixture contained 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 4 mM ATP, 0.1 mg/ml BSA, 50 μM dNTPs, 0.1 mM EDTA, 0.01% Nonidet P-40, and the indicated amounts of BER enzymes. Reaction mixtures were assembled on ice and incubated at 37 °C for 15 min. Reactions were terminated by transfer to 95 °C for 5 min in a solution containing 95% formamide and 10 mM EDTA. DNA substrates and products were separated by urea-denaturing polyacrylamide gel electrophoresis and detected by a PhosphorImager. Substrates were 32P-radiolabeled at the 5′-end of the DNA strand containing 8-oxoG.

Sizing of CAG Repeat Expansion by DNA Fragment Analysis and GeneMapper® Software—The sizes of the expansion products resulting from BER were measured by capillary electrophoresis using DNA fragment analysis and GeneMapper® version 3.7 software (Applied Biosystems, Foster City, CA) and with the generous assistance of the National Institutes of Health NIEHS Molecular Genetics Core. CAG repeats were amplified by PCR and labeled by a 6-carboxyfluorescein-tagged PCR primer that was annealed to the 5′-end flanking region of expansion products. PCR amplification was performed under the following conditions: 95 °C for 9 min, 1 cycle; 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1.5 min, 35 cycles. 6-Carboxyfluorescein-labeled PCR products were then subjected to DNA size analysis using an ABI Gene Sequencer 3730 (Applied Biosystems). Size standards were run in parallel with the PCR-amplified BER products and substrates.

Enzymatic Activity Assays—pol β DNA synthesis during BER of oxidative ssDNA breaks was measured using double-stranded oligonucleotide substrates containing (CAG)20 or random DNA sequence with 8-oxoG or THF as illustrated in Table 1. These substrates were preincubated with 1 μM OGG1 and 50 nm APE1 at 37 °C for 30 min to generate substrates for measuring pol β DNA synthesis activity. pol β activity was measured at 37 °C in a 20-μl reaction mixture containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 0.1 mM EDTA, 0.1 mg/ml BSA, 5 mM MgCl2, 50 μM dNTPs, and 0.01% Nonidet P-40. FEN1 cleavage activity on substrates with (CAG)20-THF and (CAG)20-THF flaps/ hairpins was examined at 37 °C. The 20-μl reaction mixture contained 50 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.1 mM EDTA, 0.1 mg/ml BSA, and 0.01% Nonidet P-40. APE1 incision of DNA with THF, an abasic site analog, was measured in a 20-μl reaction mixture containing 50
mm HEPES, pH 7.8, 50 mm KCl, 5 mm MgCl2, 0.1 mm EDTA, 0.1 mg/ml BSA, and 30 mm DNA substrate. DNA substrates and products were separated by 15% urea-denaturing polyacrylamide gel electrophoresis and detected by a PhosphorImager.

Probing of Hairpin Structures by T5 Exonuclease Digestion—Formation of hairpin structures by CAG repeats was probed by incubating T5 exonuclease and substrates containing (CAG)12-THF and (CAG)20-THF flap/hairpin (0.5 μm) for 30 min at 37 °C. The 10-μl reaction mixture contained 33 mm Tris acetate, pH 7.8, 66 mM potassium acetate, 10 mm magnesium acetate, and 0.5 mm dithiothreitol. DNA markers were generated by incubating 0.2 μm single-stranded oligonucleotides containing (CAG)12-THF and (CAG)20-THF with Dnase I (0.05 unit) at 25 °C for 15 min. The reaction mixture (10 μl) contained 20 mm Tris-HCl, pH 8.4, 2 mm MgCl2, and 50 mm KCl. Substrates and products were separated by 15% urea-denaturing polyacrylamide gel electrophoresis and detected by a PhosphorImager.

RESULTS

Cellular Expression of pol β and CAG Repeat Expansion during Long-patch BER of 8-OxoG in Vitro—Because pol β is a central component of BER, contributing both gap-filling synthesis and dRP lyase (35), the enzyme can influence CAG repeat stability during repair. Initially, to examine the properties of BER-related CAG repeat expansion and determine whether differences in pol β expression level could alter expansion, we studied cell extract-based BER of the oxidative DNA lesion, 8-oxoG. This lesion was embedded at the first CAG of a (CAG)20 repeat-containing DNA substrate or the same position in a random sequence-containing substrate used as a control (Fig. 1A). Experiments were conducted using cell extracts from wild-type pol β−/− mouse fibroblasts (Fig. 1A, lane 1, +/+), pol β−/− cells that do not express pol β (lane 2, −/−), and pol β−/− cells complemented with high level expression of wild-type pol β (lane 3, Comp). Fig. 1 illustrates typical results obtained with these extracts. The amount of BER products formed with both substrates varied as a function of the pol β expression level. The extract from cells expressing a high level of pol β produced more repaired products than pol β−/+ cells (Fig. 1A, lane 1), whereas pol β−/− cells generated less repaired products than pol β−/+ cells (lane 2). This is consistent with earlier results indicating a BER deficiency in the pol β−/− cells (51). Interestingly, with the random sequence-containing substrate, the repaired product corresponded to the size of the substrate only (100 nt) (Fig. 1A, left panel, indicated by arrows and size markers of 100, 115, and 130 nt), whereas with the (CAG)20 repeat-containing substrate, the repair products corresponded to the size of the substrate plus a group of slightly larger molecules (Fig. 1A, right panel, lanes 1 and 3). These larger BER products, which were ~115–130 nt, were named “expansion products.” The amount of expansion products varied with the expression level of pol β, with a moderate, significant, and large amount generated in the extracts from pol β−/−, pol β−/+−, and high level pol β-expressing cells (Fig. 1A, right panel, lane 2 and
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A

Random
Pol β cell extract
+/+  -/- Comp M

Unexpanded product (100 nt)

1 2 3

(CAG)$_{20}$

+/+  -/- Comp M

Expansion products
Unexpanded product (100 nt)

1 2 3

B

Intensity of Expansion Products (×10$^9$)

Pol β Cell Extract

Random

(CAG)$_{20}$

+C

(CAG)$_{20}$

Random

Random

C

Random (substrate)

markers

(CAG)$_{20}$-8-oxoG (substrate)

markers

(CAG)$_{20}$-8-oxoG (products)

markers

D

5' 22 nt 3' 100 nt

Random

Pol β (nM)

- 2 5 M

Expansion products
Unexpanded product

1 2 3

APE1 cleavage product

E

Random

Pol β null cell extract

- 10 20 50 M

Unexpanded product

1 2 3 4 5

APE1 cleavage product

1 2 3 4 5

(CAG)$_{20}$

Pol β (nM)

- 10 20 50 M

Expansion products
Unexpanded product

1 2 3 4 5

APE1 cleavage product

1 2 3 4 5
Quantification of the expansion products revealed that extract from wild-type and pol β-complemented cells produced more expansion products than extract from pol β−/− cells by 3- and 15-fold, respectively (Fig. 1B). The moderate amount of expansion products generated from pol β−/− cell extract could result from DNA synthesis by other DNA repair polymerases such as pol λ.

We determined the precise sizes of the expansion products after their recovery from sequencing gels using capillary electrophoresis and DNA fragment analysis. The results revealed that the expansion products contained molecules with 1-5 CAG repeat units (Fig. 1C), which was consistent with the differences in electrophoretic migration of the expansion products versus the unexpanded product (Fig. 1A). To exclude the possibility that the size measurement of these BER expansion products involved PCR artifacts, we PCR-amplified the substrates under the same conditions used for amplifying the expansion products and measured the size of this PCR-amplified material. With both the CAG repeat-containing and random sequence-containing substrates, the 100-nt DNA substrate molecule was the predominant species detected (Fig. 1C). DNA size analysis also was conducted with the BER products labeled as “unexpanded product (100 nt)” (Fig. 1A, right panel, lane 3). The size of this product was found to be 100 nt (data not shown). The results described thus far demonstrated that a size-limited CAG repeat expansion occurred during BER of 8-oxoG in cell extracts and that the expansion was stronger with a higher cellular expression of pol β. It is noteworthy that the numbers of repeat units represented in the expansions in these in vitro BER experiments were similar to those observed for Huntington disease gene CAG repeat expansions in vivo in the Huntington disease mouse model (25).3

To further explore a role for pol β in CAG repeat expansion during BER, we asked whether the expansion products could be observed in BER reactions reconstituted with purified BER enzymes and, if so, whether increasing pol β would increase their production. We reconstituted BER of 8-oxoG in the (CAG)20-containing substrate with purified human OGG1, pol β, APE1, and DNA ligase along with Fen1 (Fig. 1D). Expansion products were observed, and their amount increased with increasing concentrations of pol β (Fig. 1D). In addition, we found that production of expansion products was increased in the pol β−/− cell extract when complemented with purified pol β (Fig. 1E, right panel). With the random sequence substrate, only unexpanded product was detected (Fig. 1E, left panel).

3 A. Klungland, personal communication.
ing repair of 8-oxoG in the random DNA sequence, a single nucleotide insertion corresponded to the majority of pol β synthetic products (Fig. 2, left panel, lane 2). This indicated that pol β mainly performed one nucleotide gap-filling synthesis and exhibited weak strand displacement DNA synthesis in the context of the random DNA sequence. This was consistent with many earlier observations (44, 52, 53). In striking contrast, pol β multinucleotide synthesis occurred during synthesis on the (CAG)20-containing substrate (Fig. 2, right panel, lane 2). This synthesis did not originate from pol β strand-displacement synthesis, as the same concentration of enzyme performed only weak strand displacement synthesis on the random DNA sequence (Fig. 2, left panel, lane 2). Thus, the results are consistent with spontaneous formation of intrastrand hairpins and multinucleotide gaps during LP-BER in the context of CAG repeats. Interestingly, pol β DNA synthesis on the (CAG)20-containing substrate exhibited a pattern of periodicity with regular parting after insertion of dAMP (Fig. 2, right panel, lane 2), as illustrated by a series of synthetic DNA markers that mimic the pol β synthesis products after the insertions of dAMP (Fig. 2, right panel, lane 3). This periodicity is consistent with the idea that pol β DNA synthesis was stalled at the base of hairpins formed after strand slippage. A similar pattern of polymerase pausing on TNR templates has been reported (52–55).

**FEN1 Promotes CAG Repeat Expansion during LP-BER of Oxidative ssDNA Breaks**—FEN1 is known to be important in preventing TNR expansion during DNA replication (56). In addition, haploinsufficiency of FEN1 resulted in CAG repeat expansion in the Huntington disease mouse model (57). FEN1 also is known to be essential in LP-BER for removing groups that are resistant to pol β dRP lyase activity (56). It is possible that FEN1 also may remove CAG repeat-containing flaps and hairpins during LP-BER, which could prevent CAG repeat expansion. To examine the effects of FEN1 on CAG repeat stability during LP-BER, we used oligonucleotide substrates that contained either (CAG)20 or (CAG)25 repeat sequences with a THF, a synthetic abasic site (Fig. 3A). The THF group mimics the reduced or oxidized abasic site that would be resistant to the pol β dRP lyase activity and therefore excised by FEN1 during the LP-BER subpathway. In the absence of FEN1, complete repair products were not detected during BER reconstituted with purified APE1, pol β, and DNA ligase (Fig. 3A, lanes 1 and 3); only pol β multinucleotide insertion intermediates were observed. In contrast, the addition of FEN1 resulted in the production of repair products, including both expanded and expansion products (Fig. 3A, lanes 2 and 4). The FEN1 effect was illustrated by quantification of expansion products, as shown in the upper panel of Fig. 3C. The results suggested that the 5′-THF phosphate group had been removed by FEN1, generating nicks with 3′-OH and 5′-phosphate groups suitable for ligation, i.e., “ligatable nicks.” Thus, FEN1 played a critical role in processing the BER intermediates for production of both expanded LP-BER product and CAG repeat expansion products (Fig. 3A). The size of the expansion products was not altered by FEN1.

We also assembled reference reaction mixtures with CAG repeat substrates containing a natural 5′-dRP group (Fig. 3B). In these incubations, the pol β dRP lyase was expected to remove the 5′-dRP group. FEN1 was not expected to be involved in processing the natural 5′-dRP group or in influencing CAG repeat expansion. In the absence of FEN1, we found both unexpanded and expanded products as well as pol β multinucleotide insertion (up to five repeat units) intermediates (Fig. 3B, lanes 1 and 3). It appears that repair of the strand break intermediate in the context of CAG repeats allowed a portion of the intermediate molecules to escape SN-BER and undergo LP-BER with the associated repeat expansion. Surprisingly, addition of FEN1 stimulated the repeat expansion during repair of the substrate with the natural 5′-dRP group (Fig. 3B, lanes 2 and 4) by ~2-fold (see Fig. 3C, lower panel). In contrast, no products larger than the substrates were detected during BER of synthetic and native abasic sites with the random sequence substrates (Fig. 3, D and E). In addition, no FEN1 stimulation of CAG repeat expansion was detected in the absence of dNTPs (Fig. 3F), as expected, indicating that the FEN1 stimulatory effect was DNA synthesis-dependent. In summary, FEN1 was required for CAG repeat expansion during LP-BER of the modified 5′-sugar phosphate group. However, it also stimulated repeat expansion with a natural 5′-dRP group-containing intermediate that was subjected to the LP-BER subpathway.

**FEN1 Cleavage on Stable and Unstable CAG Repeat Hairpins**—As a flap structure-specific endonuclease (56, 58), FEN1 can cleave DNA flaps occurring in different steps of DNA replication and repair (56) leading to different DNA metabolic consequences. To characterize FEN1 cleavage on hairpin structures mimicking LP-BER within CAG repeats, oligonucleotide substrates containing a downstream 5′-THF-(CAG)12 or 5′-THF-(CAG)20 hairpin (Fig. 4) were used. The substrates containing 5′-THF-(CAG)20 and 5′-THF-(CAG)25 were selected to reflect stable hairpin and unstable hairpin/flap BER intermediates.
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FIGURE 4. FEN1 cleavage activity on THF-containing CAG repeat flaps of different length. FEN1 substrates contained a 12-repeat (A) or 20-repeat CAG flap/hairpin (B), respectively. These substrates mimic BER intermediates generated by DNA strand slippage and pol β multinucleotide gap-filling synthesis on a THF-containing BER substrate. FEN1 endonucleolytic cleavage activity was examined as described under “Experimental Procedures.” A schematic representation of the substrates is shown above the gels. A and B, Lane 1 correspond to incubation without enzyme. Lanes 2–6 correspond to reaction mixtures with 0.1, 0.5, 1, 5, and 10 nM FEN1, respectively. The configurations of 5'-THF-(CAG)_{12} containing hairpin (Fig. 4B) generated the alternate cleavage products, 5'-THF-(CAG)_{19}, 5'-THF-(CAG)_{20}, and 5'-THF-(CAG)_{20} only; the cleavage product corresponding to the full-length 5'-THF-(CAG)_{20} segment was not detected, even at the highest concentration (10 nM) of FEN1 (Fig. 4B). This suggested that 5'-THF-(CAG)_{20} adopted a hairpin loop configuration (63) that was stable enough to block FEN1 removal of the intact 5'-THF-(CAG)_{20} segment. All of the FEN1 cleavage products appeared to result from its flap cleavage activity, because the FEN1 flap cleavage-dead mutant D181A failed to produce any flap and alternate cleavage products (data not shown). The FEN1 alternate cleavage products may result from breathing at the 5'-base of the hairpin and the limited flap trimming of FEN1 at the 5'-hairpin base. This FEN1 alternate cleavage could ultimately result in a ligatable nicked hairpin intermediate for the production of expansion products.

To verify the formation of a hairpin by 5'-THF-(CAG)_{20}, we used E. coli T5 exonuclease, a single-stranded DNA-specific nuclease, to probe single-stranded regions of the molecule. It was found that T5 nuclease cleavage on the 5'-THF substrate resulted in short repeat-containing molecules plus 5'-THF-(CAG)_{19–20} as indicated in Fig. 4D. These results suggest that 5'-THF-(CAG)_{20} formed a hairpin loop structure with a 5'-THF-(CAG)_{2} flap, a large loop, and a self-base-pairing stem with a single-stranded region at the 3'-end of the stem.

respectively. We found that FEN1 cleavage on the 5'-THF-(CAG)_{12} containing hairpin resulted mainly in the 5'-THF-(CAG)_{12} product, along with small amounts of products corresponding to 5'-THF-(CAG)_{1} and 5'-THF-(CAG)_{20}, designated in Fig. 4A as “FEN1 alternate cleavage products.” These results indicate that FEN1 cleavage occurred mainly at the 3'-base of the 5'-THF-(CAG)_{12} flap, resulting in complete removal of the 5'-THF-(CAG)_{12} segment. This indicates that the entire 5'-THF-(CAG)_{12} segment was processed as a flap, and only a small portion of the substrate molecules appeared to adopt a hairpin configuration that would lead to the alternate cleavage products. On the other hand, FEN1 cleavage on 5'-THF-(CAG)_{20} containing hairpin (Fig. 4B) generated the alternate cleavage products, 5'-THF-(CAG)_{19}, 5'-THF-(CAG)_{20}, and 5'-THF-(CAG)_{20} only; the cleavage product corresponding to the full-length 5'-THF-(CAG)_{20} segment was not detected, even at the highest concentration (10 nM) of FEN1 (Fig. 4B). This suggested that 5'-THF-(CAG)_{20} adopted a hairpin loop configuration (63) that was stable enough to block FEN1 removal of the intact 5'-THF-(CAG)_{20} segment. All of the FEN1 cleavage products appeared to result from its flap cleavage activity, because the FEN1 flap cleavage-dead mutant D181A failed to produce any flap and alternate cleavage products (data not shown). The FEN1 alternate cleavage products may result from breathing at the 5'-base of the hairpin and the limited flap trimming of FEN1 at the 5'-hairpin base. This FEN1 alternate cleavage could ultimately result in a ligatable nicked hairpin intermediate for the production of expansion products.
unexpanded product was detected (Fig. 5, lanes 4 and 5), but only minor amounts of nucleotide addition intermediates were observed (Fig. 5). With the higher concentration of pol β, most of the APE1 cleavage product was converted into the unexpanded product (Fig. 5, lane 5). Thus, it appears from these results that coordination among BER enzymes occurred, whereas slippage and hairpin loop formation did not occur.

**HMGB1 Promotes CAG Repeat Expansion**—To examine a potential role of HMGB1 in modulating CAG repeat expansion during BER, we studied BER in the presence and absence of HMGB1 by making use of HMGB1 wild-type and null cell extracts. With the random sequence DNA substrate, only the 100-nt unexpanded product was detected after BER in both HMGB1+/− and HMGB1−/− cell extracts (Fig. 6A, left panel). Unexpanded product and expansion products were observed in the HMGB1+/− cell extract (Fig. 6A, right panel, lane 1) with (CAG)20-containing substrate. However, only minimal expansion products were detected in the HMGB1 null−/− cell extract (Fig. 6A, right panel, lane 2). Quantification of the expansion products formed by the two extracts revealed that the presence of HMGB1 increased production of expansion products by ~20-fold (Fig. 6B).

In additional experiments, we found that production of expansion products in the HMGB1 null cell extract was stimulated by the addition of purified HMGB1 (Fig. 6C right panel). Production of unexpanded product was stimulated also. No expansion products were observed with the random sequence substrate (Fig. 6C, left panel). These results indicated that the HMGB1 null cell extract was capable of supporting synthesis of more expansion products with the addition of HMGB1. Overall, the presence of this protein was critical for CAG repeat expansion during BER of 8-oxoG.

As HMGB1 is known to stimulate the APE1 5′-incision activity and FEN1 cleavage activity (48), HMGB1 could facilitate CAG repeat expansion by promoting production of ssDNA breaks by APE1 and/or stimulating FEN1 alternate cleavage facilitating formation of ligatable nicks. The effects of HMGB1 on APE1 and FEN1-mediated CAG repeat expansion were examined by reconstituting BER with a limiting concentration of APE1 (Fig. 7A) or FEN1 (Fig. 7B). HMGB1 strongly stimulated repeat expansion with limiting APE1 (Fig. 7A, lane 2) and slightly stimulated expansion with limiting FEN1 (Fig. 7B, lane 2). Finally, under these conditions, APE1 incision activity on THF, an AP site analog, in the context of (CAG)20 was strongly stimulated (Fig. 7C, lane 2), and the FEN1 alternate cleavage activity, producing 5′-THF-(CAG)1, was slightly stimulated by HMGB1 (Fig. 7D, lane 2).

**DISCUSSION**

We observed size-limited expansion of TNRs in a CAG repeat-containing substrate during BER of oxidative DNA base damage, both in extracts of mouse fibroblasts and in a system reconstituted with purified BER enzymes. We went on to explore mechanisms for repeat expansion that involved pol β gap-filling and FEN1 cleavage. It appeared that ssDNA breaks within CAG repeats resulted in pol β multinucleotide gap-filling DNA synthesis and FEN1 “alternate cleavage,” which promoted CAG repeat expansion. The BER cofactor, HMGB1,

Coordination among BER Enzymes and CAG Repeat Expansion—To explore a potential role of coordination among pol β, FEN1, and ligase in CAG repeat expansion was examined with a substrate containing a THF group embedded in (CAG)20 repeats. The DNA substrate is illustrated schematically above the gel. BER reactions were reconstituted with pol β (lanes 4 and 5), 5 nM FEN1, and 5 nM LIG I in the presence of dGTP and dCTP under the conditions described under “Experimental Procedures.” BER enzymes indicate a mixture of 5 nM LIG I and 5 nM FEN1. Lane 3 represents a reaction mixture in the presence of APE1 alone. Lane 1 represents substrate alone. Lane 1 (M) represents markers of 100, 115, and 130 nt. Unexpanded product and nucleotide inserted by pol β are indicated. The unexpanded product was verified by sizing analysis as described under “Experimental Procedures.” 5′-32P-Labeled DNA substrate (10 nM) was used.

**FIGURE 5.** Coordinated activities of pol β, FEN1, and DNA ligase prevent CAG repeat expansion. The role of coordination among BER enzymes in CAG repeat expansion was examined with a substrate containing a THF group embedded in (CAG)20 repeats. The DNA substrate is illustrated schematically above the gel. BER reactions were reconstituted with pol β (lanes 4 and 5), 5 nM FEN1, and 5 nM LIG I in the presence of dGTP and dCTP under the conditions described under “Experimental Procedures.” BER enzymes indicate a mixture of 5 nM LIG I and 5 nM FEN1. Lane 3 represents a reaction mixture in the presence of APE1 alone. Lane 1 represents substrate alone. Lane 1 (M) represents markers of 100, 115, and 130 nt. Unexpanded product and nucleotides inserted by pol β are indicated. The unexpanded product was verified by sizing analysis as described under “Experimental Procedures.” 5′-32P-Labeled DNA substrate (10 nM) was used.

Coordination among BER Enzymes and CAG Repeat Expansion—To explore a potential role of coordination among pol β, FEN1, and ligase in CAG repeat expansion was examined with a substrate containing a THF group embedded in (CAG)20 repeats. The DNA substrate is illustrated schematically above the gel. BER reactions were reconstituted with pol β (lanes 4 and 5), 5 nM FEN1, and 5 nM LIG I in the presence of dGTP and dCTP under the conditions described under “Experimental Procedures.” BER enzymes indicate a mixture of 5 nM LIG I and 5 nM FEN1. Lane 3 represents a reaction mixture in the presence of APE1 alone. Lane 1 represents substrate alone. Lane 1 (M) represents markers of 100, 115, and 130 nt. Unexpanded product and nucleotides inserted by pol β are indicated. The unexpanded product was verified by sizing analysis as described under “Experimental Procedures.” 5′-32P-Labeled DNA substrate (10 nM) was used.
appeared to stimulate APE1-mediated ssDNA breaks and FEN1 alternate cleavage, thereby promoting expansion in CAG repeats. Our results illustrate how the disruption of coordination between pol β/H9252 and FEN1 during LP-BER may lead to CAG repeat expansion and emphasize the critical role of FEN1 processing of the preligation hairpin intermediate.

We characterized the roles of APE1, pol β/H9252, FEN1, and HMGB1 in modulating CAG repeat stability during BER of the oxidative DNA base lesion, 8-oxoG. It is useful to summarize the requirements for these four proteins, as well as for DNA ligase in CAG repeat expansion during BER on substrates with a modified 5′-sugar phosphate (THF) (Table 2) and a natural 5′-sugar phosphate. APE1 was essential for repair with both substrates, because its 5′-incision on these abasic sites generates gapped DNA with the required 3′-OH. For BER reconstituted with these purified enzymes, pol β DNA synthesis was essential for CAG repeat expansion as expected (Table 2). However, pol β was not absolutely essential for the expansion in cell extracts, as a small amount of expansion product was detected in the pol β−/− cell extract (Fig. 1A). This indicated that other
DNA polymerases modestly complemented the function of pol β in producing unexpanded and expansion products. FEN1 was essential for repeat expansion during repair of the modified 5′-sugar phosphate (Table 2), indicating that the enzyme is required for removal of the damage that is resistant to pol β dRP lyase activity and generating a ligatable nick for hairpin ligation. In contrast, FEN1 was not required for CAG repeat expansion during repair of a natural 5′-sugar phosphate (Table 2). This finding was not surprising, as this group can be removed by pol β dRP lyase activity. HMGB1 was not required for repeat expansion in vitro when the substrate was preincised at the 5′-side of an abasic site. However, HMGB1 was crucial for repeat expansion in the cell extract (Fig. 6, right panel). This suggests that HMGB1 may be essential for stimulating APE1 5′-incision, resulting in ssDNA break intermediates required for expansion. As expected, DNA ligase was essential for ligating hairpin intermediates that lead to the repeat expansion.

Our results indicated that ssDNA breaks in CAG repeats induced by removal of 8-oxoG through OGG1 and APE1 activities are subjected to SN-BER and LP-BER, resulting in different repair consequences. SN-BER of the ssDNA break intermediate involves one nucleotide replacement accomplished by pol β single nucleotide gap-filling synthesis, pol β dRP lyase, and ligation. This BER subpathway maintains the length of the CAG repeat sequence, as there is no addition of repeat units during repair. However, ssDNA breaks may allow the repeat-containing DNA strand to undergo the slippage and formation of multinucleotide gaps and hairpins that result in pol β multinucleotide gap-filling DNA synthesis and FEN1 processing of hairpin structures during LP-BER. FEN1 alternate cleavage may further facilitate the repeat expansion by generating a trimmed hairpin and nicked intermediate for strand ligation.
FEN1 increased the amount of both unexpanded and expansion products (Fig. 3), the unexpanded product may result from coordination between FEN1 cleavage of a 5'-sugar phosphate plus one nucleotide and pol β single nucleotide gap-filling synthesis, i.e. the hit-and-run mechanism for LP-BER (44). Thus, repeat expansion may be prevented by coordinated pol β and FEN1 activities in the long-patch subpathway.

A role of FEN1 in facilitating CAG repeat expansion during LP-BER contrasts with its proposed role in preventing repeat expansion during DNA replication (57, 59–63). This could be due to differences in mechanisms involving the sequential order of formation of hairpin structures during DNA replication. It has been demonstrated that replicative DNA polymerases, such as pol δ/ε, perform strand-displacement synthesis to create a TNR flap that is captured by FEN1 before the flap can become long enough to form a stable hairpin (62). FEN1 loads

**TABLE 2**

| BER protein | (CAG)_20 TFH expansion | (CAG)_20 8-oxoG/dRP expansion |
|-------------|------------------------|-------------------------------|
| Complete BER reconstitution | + | + |
| APE1 | + | - |
| pol β | - | - |
| FEN1 | - | + |
| DNA ligase | - | + |
| HMGB1 | + | + |

**SCHEME 1.** Models illustrating CAG repeat stability modulated by coordination among BER enzymes and cofactors during LP-BER of 8-oxoG. BER of 8-oxoG in CAG repeats is initiated by OGG1 removal of the damaged base, leaving an abasic site or leaving a strand break with 5'-phosphate and 3'-blocked OH (not illustrated). APE1 incises the abasic site (5'), leaving a single nucleotide gap and a sugar phosphate flap. Repair of the single-stranded DNA break intermediate is subjected to alternative scenarios that could result in different consequences. If repair is conducted by SN-BER, where pol β fills the single nucleotide gap and removes the sugar phosphate flap with its dRP lyase activity, the repeat length will be maintained, i.e. without CAG expansion (not shown). The scheme illustrates LP-BER involving coordinated handoff of BER intermediates among pol β single nucleotide gap-filling FEN1 flap cleavage and DNA ligase that prevents CAG repeat expansion (left portion of Scheme 1, subpathway ➀). However, if this coordination is disrupted by spontaneous strand slippage and formation of hairpin loop structures during repair (right portion of Scheme 1), different consequences may occur, depending on repeat length. Short CAG repeats tend to adopt a flap configuration, as shown in subpathway ➁. FEN1 can then remove the entire short repeat flap to prevent repeat expansion. Long repeats tend to form stable hairpins, as shown in subpathway ➂. FEN1 cannot cleave at the 3'-base of the hairpin, and pol β is then forced to perform gap-filling synthesis to fill the multinucleotide gaps. The 5'-ends at the base of the hairpins then undergo realignment by reannealing to the template strand to form various sizes of CAG-containing hairpins with short 5'-flaps containing a sugar phosphate group. FEN1 is then forced to use its alternate cleavage activity to process the 5'-flap of these CAG-containing hairpins, leaving a ligatable nicked hairpin intermediate that is sealed by ligase, resulting in CAG repeat expansion. During this LP-BER, HMGB1 may facilitate CAG expansion by stimulating APE1 5'-abasic site incision activity and FEN1 alternate cleavage of short CAG-containing flaps with a sugar phosphate group at the 5'-end.
onto the flap from its 5’-end, tracks down to the 3’-base of the flap, and cleaves it (62). Thus, during DNA replication, FEN1 uses its unique tracking mechanism to prevent TNR expansion (62). During LP-BER of oxidative ssDNA breaks in CAG repeats, the weak strand displacement synthesis activity of pol β allows a TNR to undergo spontaneous strand slippage resulting in larger gaps and hairpins. pol β then fills these gaps, resulting in the addition of extra repeat units to the damaged strand. If a hairpin is intrinsically stable or stabilized by a cellular protein, FEN1 cleavage of the flap would be inhibited (63, 64). This would force FEN1 to capture and cleave a series of short CAG repeat flaps resulting from a partial breathing or realignment of the 5’-end of the hairpins. In this study, such FEN1 cleavage activity was named alternate cleavage. This type of cleavage may produce a ligatable nick along with an incompletely processed hairpin, providing the basis for repeat expansion. Because a hairpin cannot be directly ligated with the newly synthesized gap-filling product (65), breathing at the 5’-end of a hairpin and FEN1 alternate cleavage is essential for the creation of a ligatable nick and completion of CAG repeat expansion.

Our results suggest that CAG repeats were expanded during LP-BER of 8-oxoG involving pol β gap-filling synthesis and FEN1-mediated alternate cleavage of short flaps at the 5’-end of hairpin structures. The results also predict that repeat expansion would be prevented in this subpathway when sequential handoff of BER intermediates between pol β and FEN1, i.e. hit-and-run coordination, is reinforced. This was illustrated by our results showing that unexpanded product formation was maintained when the BER incubation was performed in the presence of dGTP and dCTP only (Fig. 5). These results suggested that BER coordination was reinforced under conditions of limited usage of dNTP, i.e. to dGTP and dCTP. Thus, as illustrated in Scheme 1, subpathway 1, during LP-BER of 8-oxoG within a CAG repeat, if the pol β single nucleotide gap-filling product is directly passed to FEN1, FEN1 will remove one nucleotide attached to the sugar phosphate to create another 1-nucleotide gap that can subsequently be passed to pol β and then to DNA ligase for ligation. In this case, the coordinated BER enzyme activities can effectively accomplish repair before the CAG repeat-containing strand can undergo slippage to form hairpins.

Scheme 1 further illustrates how coordination among pol β, FEN1, HMGB1, and DNA ligase could govern the stability of CAG repeats during LP-BER of 8-oxoG. In the case of strand slippage, the effect of repeat length on CAG repeat expansion is illustrated in subpathways 2 and 3 (Scheme 1). The size of the expansion products may be determined by both the size and stability of the hairpin loops (Scheme 1, subpathway 3). The LP-BER cofactor, HMGB1, can stimulate expansion by modulating the APE1 5'-incision for generating ssDNA breaks (Figs. 6 and 7) and the FEN1 processing of hairpins. Other BER cofactors, such as proliferating cell nuclear antigen (66) and Werner syndrome protein (67), that stimulate FEN1 could also stimulate FEN1 alternate cleavage. In contrast, coordination between FEN1 and LP-BER cofactors that can unwind hairpin structures, such as Werner syndrome protein helicase, may facilitate removal of hairpins and thereby prevent repeat expansion.

FEN1 and Werner syndrome protein can physically interact with each other; however, destabilization of hairpins by Werner syndrome protein helicase activity requires replication protein A (68). It is conceivable that protein-protein interactions and coordination among FEN1, Werner syndrome protein, and replication protein A destabilizes hairpin structures and, therefore, facilitates FEN1 cleavage, preventing expansion. As efficient SN-BER is thought to be mediated by multiple DNA-protein and protein-protein interactions and coordination among APE1, pol β, x-ray repair complement group 1, and polynucleotide kinase, it appears that disruption of coordination among these enzymes and cofactors would favor CAG repeat strand slippage, resulting in LP-BER and repeat expansion. The mechanisms by which these macromolecular interactions operate in preventing or promoting CAG repeat expansion during BER need to be elucidated.

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