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Proliferating cell nuclear antigen prevents trinucleotide repeat expansions by promoting repeat deletion and hairpin removal

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Abstract

DNA base lesions and base excision repair (BER) within trinucleotide repeat (TNR) tracts modulate repeat instability through the coordination among the key BER enzymes DNA polymerase β , flap endonuclease 1 (FEN1) and DNA ligase I (LIG I). However, it remains unknown whether BER cofactors can also alter TNR stability. In this study, we discovered that proliferating cell nuclear antigen (PCNA), a cofactor of BER, promoted CAG repeat deletion and removal of a CAG repeat hairpin during BER in a duplex CAG repeat tract and CAG hairpin loop, respectively. We showed that PCNA stimulated LIG I activity on a nick across a small template loop during BER in a duplex (CAG)₂₀ repeat tract promoting small repeat deletions. Surprisingly, we found that during BER in a hairpin loop, PCNA promoted reannealing of the upstream flap of a double-flap intermediate, thereby facilitating the formation of a downstream flap and stimulating FEN1 cleavage activity and hairpin removal. Our results indicate that PCNA plays a critical role in preventing CAG repeat expansions by modulating the structures of dynamic DNA via cooperation with BER enzymes. We provide the first evidence that PCNA prevents CAG repeat expansions during BER by promoting CAG repeat deletion and removal of a TNR hairpin.

Keywords

Proliferating cell nuclear antigen (PCNA); Base excision repair (BER); Trinucleotide repeats; DNA repair; DNA polymerase β (pol β); Flap endonuclease 1 (FEN1)

1. Introduction

Over 40 human neurodegenerative diseases are caused by trinucleotide repeat (TNR) expansions, including Huntington's disease (CAG/CTG), myotonic dystrophy (CTG/CAG),

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Conflict of interest

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and Friedreich's ataxia (GAA/TTC), among others [1,2]. This instability results from the formation of non-B form DNA structures such as hairpins, triplexes, sticky DNA, and tetraplexes [3–5] during DNA replication and repair, recombination and gene transcription [3,6–8]. TNR tracts contain tandem purines that are hotspots for the formation of oxidized lesions [9], and repeated cycles of base damage and inefficient DNA base excision repair (BER) lead to cumulative TNR expansions through a “toxic oxidation cycle” that could subsequently result in the onset of disease [2,10]. BER of oxidized base lesions in a TNR tract has been shown to modulate repeat instability [5,11–15] as the result of a loss of coordination between BER proteins and cofactors [11–14] caused by the formation of secondary structures. We have recently shown that oxidative and alkylated DNA damage within TNR tracts induce large repeat deletions and small expansions through BER, indicating that DNA base lesions and BER also play an important role in mediating TNR deletions [12,15–17]. We have further demonstrated that the location of a lesion within a duplex TNR tract determines whether a TNR deletion or expansion occurs as a result of the formation of secondary structures in either the damaged or template strand [15]. A base lesion located at the 5′-end of a TNR tract induced expansions as a result of formation of a large hairpin in the damaged strand which interrupts efficient flap cleavage by flap endonuclease 1 (FEN1). A lesion located in the middle of the repeat tract induced deletions as a result of formation of a template hairpin that is bypassed by DNA polymerase β (pol β) [15]. Furthermore, we found that a bulky oxidized base lesion, 5′, 8-cyclo-2′-deoxyadenosine (cdA), in a (CAG)₂₀ template strand caused CTG repeat deletions during BER and DNA lagging strand maturation by inducing formation of a small CAG repeat loop that was subsequently skipped over by pol β lesion bypass synthesis [17]. We showed that the deletions were dependent on the location of the base lesion [17]. Moreover, we have found that BER of a base lesion located in the loop of a TNR hairpin leads to hairpin removal and the prevention or attenuation of expansions [18,19]. This occurs when the hairpin is converted into a double-flap intermediate, containing an upstream 3′-flap and a downstream 5′-flap, as a result of APE1 5′-incision of the abasic site. Subsequently, the double-flap intermediates can be resolved by the coordinated actions of FEN1 with the 3′-5′ endonuclease Mus81/Eme1 and the 3′-5′ exonuclease activity of AP endonuclease 1 (APE1), which resolve the downstream and upstream flaps of the intermediates, respectively [18,19]. This indicates that the type of base lesion and its location are critical in determining whether BER facilitates or prevents TNR instability.

Efficient BER is mediated by functional coordination among the repair enzymes as well as their coordination with BER cofactors. During long-patch BER, pol β gap-filling synthesis coordinates with FEN1 flap cleavage via a “Hit and Run” mechanism in which pol β gap-filling synthesis creates a flap for FEN1 cleavage that in turn generates an additional single-nucleotide gap which is then filled in by pol β [20]. Efficient BER also relies on coordination of the BER core enzymes with repair cofactors such as poly(ADP-ribose) polymerase1 (PARP1) [21], proliferating cell nuclear antigen (PCNA) [22–25], X-ray repair cross-complementing 1 (XRCC1) [26–28], and high mobility group Box 1 (HMGB1) [29]. PARP1 acts as a nick sensor protein and stimulates FEN1 flap cleavage, thereby promoting pol β DNA synthesis [21]. XRCC1 physically interacts with pol β and DNA ligase III (LIG III) to enhance the efficiency of LIG III during single-nucleotide BER [26–28]. HMGB1 can

stimulate pol β DNA synthesis and FEN1 flap cleavage, increasing the efficiency of long-patch BER [14,29]. Disruption of the coordination between pol β and FEN1 by a CAG repeat hairpin leads to pol β multi-nucleotide gap-filling synthesis and low efficiency of long-patch BER resulting in CAG repeat expansions [13]. Thus, efficient BER that is mediated by BER protein-protein interactions and coordination plays a crucial role in preventing TNR expansions. The role of repair cofactors in maintaining trinucleotide repeat and greater genomic stability has yet to be explored.

PCNA acts as the sliding clamp to increase the processivity of mammalian replication polymerases. It can also interact with other enzymes and proteins involved in DNA replication, DNA repair, and cell cycle control [30–35]. PCNA is a toroidal homotrimeric protein that encircles double-stranded DNA and slides along the duplex DNA. It can interact with DNA glycosylases [36], pol β [32], FEN1 [22,33,37–39], and DNA ligase I (LIG I) [23,40], and has been found to localize to the nucleus in response to treatment with DNA alkylating agents such as methylmethanesulfonate (MMS) [41], indicating its crucial role in mediating efficient BER [24,25,34]. PCNA and FEN1 physically interact through the PCNA-interacting protein (PIP) box at the C-terminus of FEN1 and a hydrophobic cleft on the proximal face of the PCNA trimer [42,43]. The interaction occurs after FEN1 loads onto the free 5'-end of the flap and tracks down to the base. PCNA then slides along the duplex from upstream of the flap to interact with FEN1 at the flap base [33]. This stabilizes FEN1 binding to the substrate [33] and stimulates the FEN1 flap cleavage activity [37,38], thereby facilitating efficient processing of Okazaki fragments and long-patch BER [22,33,44]. It has been shown that disruption of the interaction between yeast PCNA and the yeast FEN1 homolog, Rad27, as well as that between yeast PCNA and LIG I, increases CAG repeat expansions and deletions [45], indicating that the interactions between PCNA and FEN1 and LIG I are critical in the maintenance of TNR stability. Moreover, mutations of PCNA that disrupt its interaction with FEN1 have been shown to be associated with DNA repair disorders with neurodegenerative symptoms [46]. The results suggest that PCNA plays a crucial role in the maintenance of genome stability. We further hypothesized that PCNA prevents TNR expansions by coordinating with FEN1 and other repair enzymes during BER in a TNR tract, and in doing so, prevents TNR instability and facilitates maintenance of genome stability. To test this hypothesis, we explored the effects of PCNA on TNR instability during BER in a duplex CAG repeat tract and in the loop of a CAG repeat hairpin. For the first time, we found that PCNA prevented CAG repeat expansions during BER in a (CAG)₂₀ duplex and (CAG)₇ and (CAG)₁₄ hairpins by promoting CAG repeat deletions and removal of a CAG repeat hairpin. We further demonstrated that the formation of secondary structures in a CAG repeat tract determined whether PCNA can modulate CAG repeat instability during BER in a CAG duplex or hairpin by modulating the activities of BER enzymes. We provide the first evidence that a DNA repair cofactor such as PCNA can play a significant role in maintaining genome stability during repair in a CAG repeat tract.

2. Materials and methods

2.1. Materials

The DNA oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA). Deoxynucleotide 5'-triphosphates (dNTPs) were from Fermentas (Glen Burnie, MD, USA). T4 polynucleotide kinase and terminal deoxynucleotidyltransferase were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Radionucleotides [γ - ^{32}P] ATP (6000 mCi/mmol) and Cordycepin 5'-triphosphate 3'-[α - ^{32}P] (5000 mCi/mmol) were purchased from PerkinElmer Inc. (Boston, MA, USA). Micro Bio-Spin 6 chromatography columns were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All standard chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Thermo Fisher Scientific (Waltham, MA, USA). PCNA was purchased from Enzymax (Lexington, KY, USA). Pol β was a generous gift from Dr. Samuel H. Wilson at the National Institute of Environmental Health Sciences/National Institutes of Health. APE1, FEN1, and LIG I were expressed in *Escherichia coli* and purified as described below.

2.2. Oligonucleotide substrates

Double-flap oligonucleotide substrates were prepared as described previously, with slight modifications [18]. Briefly, substrates mimicking the double-flap intermediates with a 5'-tetrahydrofuran (THF) residue were constructed by annealing an upstream primer containing a 3'-(CAG)₄ or (CAG)₆ flap and a downstream primer containing a 5'-(CAG)₃ or (CAG)₆ flap with the template strand containing (CTG)₇ or (CTG)₁₀, respectively, at a molar ratio of 1:3:3. Substrates containing a (CAG)₂₀ duplex with a THF in the first or tenth repeat unit were constructed by annealing a (CAG)₂₀-containing oligonucleotide containing a THF lesion with the complementary template strand containing (CTG)₂₀ at a ratio of 1:3. A substrate mimicking a duplex (CAG)₂₀ repeat intermediate with an abasic lesion in the first repeat pre-cleaved with APE1 and FEN1 was constructed by annealing an upstream primer containing a 3'-CA and a downstream primer containing a phosphorylated AG(CAG)₁₈ to a template strand containing (CTG)₂₀ at a ratio of 1:3:3. Oligonucleotide sequences are listed in Supplementary Table 1. Substrates were labeled with ^{32}P at the 5'- or 3'-end of the damaged strand, upstream or downstream primer, or template strand, as indicated.

2.3. Protein expression and purification

FEN1 and APE1 were purified as described previously [19]. Briefly, FEN1 was expressed in *E. coli* BL21(DE3). Two liters of lysogeny broth (LB) medium cultures were incubated at 37 °C at 225 rpm until OD₆₀₀ reached 0.6. The protein expression was induced with 1 mM IPTG for 3.5 h and harvested by centrifugation at 2600 rpm for 45 min at 4° C. Cells were lysed with a French press cell disruptor (Glen Mills, Clifton, NJ, USA) in lysis buffer which contained 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.5, 30 mM KCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.5% inositol. The supernatant was subjected to purification through a Sepharose Q, CM sepharose and phenyl sepharose column sequentially operated by an AKTA Fast Protein Liquid Chromatography system (FPLC) (GE Healthcare, Piscataway, NJ, USA). The peak fractions were combined and dialyzed into buffer containing 30 mM

HEPES, pH 7.5, 30 mM KCl, 0.5% inositol, and 1 mM PMSF. Samples were then loaded onto a 1-ml Mono-S column (GE Healthcare, Piscataway, NJ, USA), and eluted using a linear gradient of KCl (30 mM–2 M). Purified FEN1 was aliquoted and frozen at –80 °C until further use.

APE1 was expressed in *E. coli* BL21(DE3). Two liters of LB medium cultures were incubated at 37 °C at 225 rpm until OD₆₀₀ reached 0.6. The APE1 expression was induced by 0.5 mM IPTG for 3.5 h. Cells were harvested by centrifugation at 2500 rpm for 30 min at 4 °C. Cells were lysed in lysis buffer, which contained 50 mM HEPES, pH 7.5, 30 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, and 1 mM PMSF. The supernatant was subjected to sequential purification by Q sepharose, CM sepharose, Mono-S and phenyl sepharose columns operated by an AKTA FPLC. Purified APE1 was aliquoted and frozen at –80 °C until further use.

LIG I was expressed in *E. coli* BL21(AI). Six liters of LB medium were inoculated with one colony each of the transformed BL21(AI) cells and were incubated overnight without shaking. The cells were then incubated at 37 °C at 225 rpm until OD₅₉₅ reached 0.6. LIG I expression was induced by 1 mM IPTG for 24 h at 15–18 °C. Cells were harvested by centrifugation at 2500 rpm for 30 min at 4 °C. Cell pellets were collected and lysed in lysis buffer, which contained 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Nonidet P-40, 1 mM PMSF, and 1 tablet of cComplete protease inhibitors (Roche, Indianapolis, IN) and subjected to French Press at 150,000 PSI. The cell lysates were centrifuged at 12,000 rpm for 30 min at 4 °C. The supernatant was loaded onto a 20-ml P11 Phosphocellulose column, with fractions eluted using a linear gradient of NaCl (50 mM–600 mM). The peak fractions were combined and dialyzed into buffer containing 30 mM HEPES, pH 7.0, 30 mM KCl, 0.1% inositol, and 1 mM PMSF. Samples were then loaded onto a 10-ml Q sepharose column and eluted using a linear gradient of KCl (30 mM–2 M). Peak fractions were combined and dialyzed into buffer containing 50 mM Tris–HCl, pH 8.0, 500 mM NaCl, 7 mM 2-mercaptoethanol, 10 mM imidazole, 0.5% inositol, and 1 mM PMSF. Samples were loaded onto a 4-ml Nickel-Nitrilotriacetic acid (Ni-NTA) column, with fractions eluted using a linear gradient of imidazole (10 mM–600 mM). Purified LIG I was aliquoted and frozen at –80 °C until further use.

2.4. Reconstituted BER assay

In vitro BER of an abasic site analog, THF, in a (CAG)₂₀ duplex or at the 5'-end of the downstream primer of the double-flap substrate was carried out by incubating 25 nM substrate with the indicated concentrations of APE1, FEN1, LIG I, pol β, and PCNA. Substrates containing a (CAG)₂₀ duplex with a THF residue were pre-incubated with APE1 at 37 °C for 30 min and then subjected to BER reactions. All reactions were carried out in reaction buffer containing 30 mM HEPES, pH 7.8, 40 mM KCl, 5% glycerol, and 0.1 mg/ml bovine serum albumin (BSA), with 5 mM MgCl₂, 2 mM ATP, and 50 μM dNTPs. The 20 μl BER reaction mixtures were incubated at 37 °C for 15 min, and reactions were terminated by the addition of 20 μl stopping buffer containing 95% formamide and 10 mM EDTA. Reaction mixtures were then denatured at 95 °C for 10 min and separated by 15% urea-denaturing polyacrylamide gel electrophoresis. Substrates and products were detected and

analyzed using a Pharos FX Plus PhosphorImager from Bio-Rad Laboratories (Hercules, CA, USA). All experiments were done in triplicate.

2.5. BER enzymatic activity assay

The activities of FEN1 flap cleavage, pol β DNA synthesis, and LIG I on all substrates were measured by incubating 25 nM substrates with the indicated concentrations of FEN1, pol β , LIG I, and PCNA in reaction buffer containing 30 mM HEPES, pH 7.8, 40 mM KCl, 5% glycerol, and 0.1 mg/ml bovine serum albumin (BSA), with 5 mM $MgCl_2$, 2 mM ATP, and 50 μ M dNTPs. The 20 μ l BER reaction mixtures were incubated at 37 °C for 15 min, and reactions were terminated by the addition of 20 μ l stopping buffer. Reaction mixtures were then denatured at 95 °C for 10 min and separated by 15% urea-denaturing polyacrylamide gel electrophoresis. Substrates and products were detected and analyzed using a Pharos FX Plus PhosphorImager from Bio-Rad Laboratories.

2.6. Probing of hairpin structures and flaps by S1 nuclease digestion

The formation of hairpin and flap structures formed by the duplex and double-flap substrates was probed using S1 nuclease as described previously, with slight modifications as described [18]. Briefly, substrates (25 nM) were incubated with the indicated concentrations of S1 nuclease in its reaction buffer at 37 °C for 1, 5, 10, and 15 min in the absence and presence of PCNA (100 nM). Reaction mixtures were then subjected to proteinase K digestion at 55 °C for 30 min to remove the S1 Nuclease. Reactions were stopped with 15 μ l of stopping buffer. Reaction mixtures were then denatured at 95 °C for 10 min and separated by 18% urea-denaturing polyacrylamide gel electrophoresis. Substrates and products were detected and analyzed using a Pharos FX Plus PhosphorImager from Bio-Rad Laboratories.

3. Results

3.1. PCNA stimulates CAG repeat deletion during BER of an abasic site in a duplex (CAG)₂₀ repeat tract in the absence of pol β

Since our previous studies have shown that TNR instability induced by a base lesion is governed by the location of a lesion in a duplex TNR tract during BER [15,17], we initially determined whether PCNA can facilitate efficient BER of an abasic lesion located at the first (5'-end) and tenth (middle) repeat of a duplex (CAG)₂₀ repeat tract to prevent repeat expansions. We reconstituted BER using the substrates containing a duplex (CAG)₂₀ tract with an abasic lesion, the THF residue, at the 5'-end (Fig. 1, left panel) or in the middle (Fig. 1, right panel). We found that in the absence of pol β , BER of the base lesion at both locations resulted in the production of a repaired product with a one repeat deletion (Fig. 1, lanes 4–6 and lanes 13–15). The amount of the deletion products was increased in the presence of 50 nM and 100 nM PCNA (Fig. 1, lanes 5–6 and lanes 14–15). The presence of pol β resulted in the production of the unexpanded product with the substrates containing an abasic lesion at either the 5'-end or the middle of the (CAG)₂₀ repeat tract (Fig. 1, lane 7 and lane 16). However, under these conditions, PCNA at 50 nM and 100 nM did not significantly affect the amount of the repaired products (Fig. 1, lanes 8–9 and lanes 17–18). The results indicate that PCNA stimulated CAG repeat deletions in the absence of pol β during BER of an abasic lesion in a duplex (CAG)₂₀ tract.

3.2. Formation of secondary structures in a duplex (CAG)₂₀ repeat tract can be modulated by PCNA

We have previously shown that the formation of template hairpins mediates repeat deletions and expansions during BER in the context of a TNR tract [12,15,17]. To further test whether the secondary structures formed in a duplex (CAG)₂₀ repeat tract can be modulated by PCNA, we initially probed for the formation of secondary structures in the template strand of the (CAG)₂₀ substrate with an abasic lesion at the 5'-end (Fig. 2A) or the middle (Fig. 2B) of the repeat tract using S1 nuclease that specifically cleaves single-stranded DNA. For the damage at the 5'-end, S1 nuclease resulted in cleavage products of 19 nt–23 nt in the absence and presence of PCNA (Fig. 2A, lanes 3–6 and lanes 10–13) indicating that a small loop containing one CTG formed on the template strand (Fig. 2A, the scheme below the gels) and PCNA failed to alter the loop structure. S1 nuclease cleavage on the template strand of the substrate with an abasic lesion in the middle resulted in products with 46 nt, 49 nt and 51 nt (Fig. 2B, lanes 3–6) indicating the formation of a (CTG)₂ loop on the template strand. In the presence of PCNA, S1 nuclease cleavage on the template strand resulted in products with 51 nt and 52 nt (Fig. 2B, lanes 10–13), indicating the formation of a loop with one CTG. S1 nuclease cleavage on the upstream strand of the damaged strand of the substrate resulted in cleavage products of 44nt–48nt (Fig. 2C, lanes 3–6) in the absence of PCNA, indicating the formation of a (CAG)₂ flap. However, in the presence of PCNA, S1 nuclease only resulted in a 48nt product (Fig. 2C, lanes 10–13), indicating the formation of a flap with one CAG. The results indicated that PCNA promoted reannealing of the upstream repeat flap to the template strand, presumably by sliding towards the downstream strand. The results also indicated that PCNA altered the structure of a (CTG)₂ loop on the template strand and converted it to a smaller (CTG)₁ loop.

3.3. PCNA does not stimulate FEN1 flap cleavage activity during BER in a duplex (CAG)₂₀ repeat tract

PCNA has been shown to directly interact with FEN1 and stimulate its flap cleavage during BER [37,38]. To determine whether PCNA can also stimulate FEN1 flap cleavage activity on a duplex (CAG)₂₀ repeat tract during BER, we examined the effects of PCNA on FEN1 cleavage during BER in the context of the (CAG)₂₀ tract with an abasic lesion located at the 5'-end (Fig. S1, left panel) or in the middle (Fig. S1, right panel) of the repeat tract. Here, 5 nM FEN1 was used to determine the stimulatory effect of PCNA on FEN1 flap cleavage on duplex CAG repeat substrates. This is because APE1 cleavage of the (CAG)₂₀ duplex allowed formation of a flap intermediate with a big gap, which is a poor substrate for FEN1 cleavage, and necessitates that a higher concentration of FEN1 be used. This was supported by our results showing that low concentrations of FEN1 (<5 nM) did not result in any cleavage products, either in the presence or absence of PCNA (data not shown). For both substrates, we found that in the absence and presence of 50 nM and 100 nM PCNA, similar amounts of cleavage products were generated by 5 nM FEN1 in the absence of pol β (Fig. S1, compare lanes 5–6 with lane 4 and lanes 15–16 with lane 14). Similarly, in the presence of pol β, 50 nM and 100 nM PCNA did not significantly alter the amount of FEN1 cleavage products (Fig. S1, compare lanes 9–10 with lane 8 and lanes 19–20 with lane 18). The results indicate that PCNA did not stimulate FEN1 flap cleavage activity during BER in a duplex (CAG)₂₀ tract.

3.4. PCNA does not alter pol β synthesis activity during BER of an abasic site in a duplex (CAG)₂₀ repeat tract

PCNA has previously been shown to physically interact with pol β [32]. To determine whether PCNA can stimulate pol β synthesis during BER in a duplex (CAG)₂₀ tract, we examined pol β DNA synthesis on the (CAG)₂₀ containing substrates (Supplementary Fig. S2) in the absence and presence of 50 nM and 100 nM PCNA. We found that with 2 nM, 5 nM, and 10 nM pol β , similar amounts of pol β DNA synthesis products were formed in the absence and presence of PCNA (Supplementary Fig. S2, compare lanes 5–6 with lane 4, lanes 8–9 with lane 7, lanes 11–12 with lane 10, lanes 17–18 with lane 16, lanes 20–21 with lane 19, and lanes 23–24 with lane 22). Thus, the results indicated that PCNA did not affect pol β DNA synthesis during BER in a duplex (CAG)₂₀ tract. This further indicates that the PCNA-pol β interaction did not alter BER in a duplex (CAG)₂₀ tract.

3.5. PCNA stimulates LIG I in the context of a small loop on the template strand of a duplex (CAG)₂₀ repeat tract in the absence of pol β

Because PCNA stimulated the production of deletion products in the absence of pol β during BER in a duplex (CAG)₂₀ tract (Fig. 1), but failed to stimulate FEN1 and pol β (Supplementary Figs. S1 and S2), it is possible that the stimulatory effect of PCNA on small repeat deletion may result from its stimulation of LIG I activity across a small bubble or hairpin in the template strand, as it stimulates the activity of LIG I in duplex DNA [23]. To test this, we examined the effect of 50 nM and 100 nM PCNA on the activity of LIG I (1 nM and 5 nM) in the absence and presence of pol β on the substrate with a small (CTG)₁ repeat loop in the template strand. The substrate mimics the intermediate with a nick across from a small (CTG)₁ loop on the template strand at the 5'-end that is generated by FEN1 cleavage of the sugar phosphate of an abasic lesion and adjacent nucleotide. The results showed that 50 nM and 100 nM PCNA significantly increased the amount of ligation product resulting from 1 nM and 5 nM LIG I activity in the absence of pol β (Fig. 3, compare lanes 3–4 with lane 2 and lanes 6–7 with lane 5). This indicates that PCNA stimulated LIG I to seal a nick across a small CTG loop, facilitating the formation of small deletion products during BER in a duplex (CAG)₂₀ repeat tract. PCNA did not significantly stimulate LIG I activity in the presence of pol β , although a little more ligation product was generated in the presence of 1 nM LIG I with PCNA than without PCNA (compare Fig. 3 lanes 10–11 with lane 9 and lanes 13–14 with lane 12). The results indicate that PCNA stimulated LIG I across from a template bubble only in the absence of pol β .

3.6. PCNA stimulates the processing of a double-flap intermediate during BER in a CAG hairpin loop

Our previous studies have shown that during BER of an abasic lesion in the loop region of a CAG repeat hairpin, APE1 5'-incision converts the hairpin into a double-flap intermediate with an upstream 3'-flap and a downstream 5'-flap [18,19]. This intermediate was then processed by the coordinated actions among FEN1flap cleavage, Mus81/Eme1 3'-5' endonuclease [18] and APE1 3'-5' exonuclease [19], leading to removal of the hairpin and prevention of TNR expansions. To further test whether PCNA can also facilitate the removal of the hairpin by stimulating FEN1 flap cleavage activity for processing of the double-flap

intermediate, we reconstituted BER in the absence and presence of PCNA with a substrate containing an upstream 3'-(CAG)₄ flap and a downstream 5'-(CAG)₃ flap with a 5'-THF residue, as well as with a substrate containing a (CAG)₆ flap on both the upstream and downstream strands with a 5'-THF residue. These substrates mimic the double-flap intermediates which are formed during BER of an abasic lesion in a (CAG)₇ and (CAG)₁₂ hairpin, respectively. On the substrate containing short (CAG)₃/(CAG)₄ flaps, we found that the presence of 50 nM and 100 nM PCNA significantly increased the amount of the unexpanded repaired product that has the same length as the template strand without and with pol β (10 nM) (Fig. 4, left panel, compare lanes 6–7 with lane 5 and lanes 9–10 with lane 8). On the substrate containing long (CAG)₆/(CAG)₆ flaps, PCNA (50 nM and 100 nM) did not significantly affect the production of the unexpanded repaired product in the absence of pol β (Fig. 4, right panel, compare lanes 16–17 with lane 15). However, in the presence of 10 nM pol β, 50 nM and 100 nM PCNA resulted in a slight increase in the amount of unexpanded repaired product (Fig. 4, right panel, compare lanes 19–20 with lane 18). The results indicate that PCNA significantly promoted the resolution of the short double-flap intermediate and moderately facilitated the removal of the long double-flap intermediate leading to the removal of the CAG repeat hairpin during BER.

3.7. PCNA stimulates FEN1 flap cleavage activity on a double-flap intermediate of hairpin repair

PCNA has been shown to stimulate FEN1 flap cleavage activity to facilitate DNA lagging strand maturation [33,37] and long-patch BER [22,38]. To determine whether PCNA also promotes the resolution of the double-flap intermediate and subsequent hairpin removal by stimulating FEN1 5'-flap cleavage of the double-flap intermediate, we examined FEN1 cleavage activity on the shorter (CAG)₃/(CAG)₄ double-flap substrate and the longer (CAG)₆/(CAG)₆ double-flap substrate in the absence and presence of PCNA (Fig. 5). We found that PCNA (50 nM and 100 nM) significantly increased the amount of FEN1 cleavage of the downstream flap of the short double-flap substrate independent of pol β (Fig. 5, left panel, compare lanes 4–5 with lane 3 and lanes 8–9 with lane 7). However, the same concentrations of PCNA only slightly stimulated FEN1 cleavage of the downstream flap of the long double-flap substrate (Fig. 5, right panel, compare lanes 13–14 with lane 12 and lanes 17–18 with lane 16). The results indicate that PCNA mainly stimulated FEN1 flap cleavage activity on the short double-flap intermediate during BER in a small CAG repeat hairpin, promoting hairpin removal and the formation of unexpanded repaired products. The weaker stimulatory effect of PCNA on FEN1 cleavage of the longer flap likely results from the longer upstream flap forming a greater obstacle to the advance of PCNA along the duplex as compared to a shorter flap. The results further suggest that PCNA managed to slide toward the downstream flap to interact with FEN1, thereby stimulating its flap cleavage activity. We then hypothesized that this could result from reannealing of the upstream strand to the template strand as the upstream strand could be pushed by the sliding of PCNA along the duplex toward the downstream. To test this, we examined the formation of the flap or secondary structures in the upstream strand of the double-flap substrates in the absence and presence of PCNA (Fig. 6). We found that in the absence of PCNA, S1 nuclease cleavage on the upstream strand of the short double-flap substrate led to the cleavage products of 25 nt, 26 nt, 27 nt, 28 nt, 30 nt, 31 nt, 34 nt, and 35 nt (Fig. 6A, left panel, lanes 2–5 and

quantitative results shown in the bar chart below the gels), indicating that the upstream strand adopted a (CAG)₄ repeat flap (Fig. 6A, the scheme below the gels). In the presence of PCNA, S1 nuclease failed to produce any cleavage products at 1 min (Fig. 6A, lane 8). However, at 5 min, 10 min and 15 min, the nuclease cleavage only resulted in the products containing 27 nt, 30 nt, 31 nt, and 37 nt (Fig. 6A, right panel, lanes 9–11 and quantitative results shown in the bar chart below the gels). The reduced S1 nuclease cleavage (Fig. 6A, quantitative results shown below the gels) indicates that the presence of PCNA promoted the reannealing of the upstream strand to the template strand through PCNA sliding toward the downstream strand. This subsequently resulted in the formation of a substrate with an annealed upstream and longer downstream flap allowing PCNA to interact with FEN1 and stimulate its cleavage activity for removing a long CAG repeat flap. This is supported by the results in Fig. 5, which indicate that PCNA was able to stimulate FEN1 on the double-flap substrates, which would require PCNA to advance past the upstream flap. Similarly, in the absence of PCNA, S1 nuclease cleavage on the upstream strand of the long (CAG)₆/(CAG)₆ double-flap substrate resulted in the products with 22 nt, 25 nt, 28 nt, 31 nt, 34 nt, 37 nt, 40 nt, 43 nt, and 46 nt (Fig. 6B, left panel lanes 3–5 and the bar chart below the gels), indicating that the upstream strand formed a long (CAG)₇ flap. In the presence of PCNA, the nuclease cleavage resulted in no cleavage products after 1 min of incubation with S1 nuclease (Fig. 6B, lane 8), and only led to a small amount of products with 22–47 nt after 5–15 min of incubation (Fig. 6B, right panel, lanes 9–11 and the bar chart). At all time intervals, large amounts of residual substrate remained uncleaved by the S1 nuclease, indicating that the majority of the substrate was annealed and resistant to cleavage by S1 nuclease. The quantitative data showed that the percentage of S1 nuclease cleavage products was significantly decreased in the presence of PCNA for both the (CAG)₃/(CAG)₄ and (CAG)₆/(CAG)₆ double-flap substrates (Fig. 6, compare right side to left side). For example, for the (CAG)₆/(CAG)₆ double-flap substrate, in the absence of PCNA, up to 25% of the upstream flap of the long double-flap substrate was cleaved by S1 nuclease (Fig. 6B, the panel on the left and the graph below the gel), whereas in the presence of PCNA, only up to 5% of the upstream strand of the substrate was cleaved (Fig. 6B, the panel on the right and the graph below the gel). This indicates that in the absence of PCNA, the upstream strand formed into a long double-flap which was sensitive to S1 cleavage. However, the presence of PCNA pushed the upstream flap to reanneal with its template forming a duplex DNA segment that was insensitive to S1 cleavage. Overall, our results indicate that PCNA sliding on the duplex DNA pushed the upstream strand to reanneal to the template strand (Fig. 6B, the scheme below the gels). Moreover, we found that S1 nuclease failed to make any cleavage on the template strand of the double-flap substrates (Supplementary Fig. S3), indicating that no secondary structures formed on the template strand to block PCNA sliding.

3.8. PCNA slightly facilitates pol γ synthesis activity on the double-flap intermediate

Since our results indicate that PCNA sliding pushed the upstream strand of the double-flap substrates to reanneal to the template strand (Fig. 6), this may further create more 3'-termini for pol β DNA synthesis. Thus, it is possible that PCNA may stimulate pol β DNA synthesis during BER in a hairpin by altering the dynamics of the double-flaps. To test this, we examined pol β DNA synthesis on the (CAG)₃/(CAG)₄ and (CAG)₆/(CAG)₆ double-flap substrates (Fig. 7) in the absence and presence of 50 nM and 100 nM PCNA. We found that

PCNA (50 nM and 100 nM) increased the amount of DNA synthesis products on the (CAG)₃/(CAG)₄ substrate generated by pol β at both 2 nM and 5 nM (Fig. 7, left panel, compare lanes 4–5 with lane 3 and lanes 7–8 with lane 6). For the (CAG)₆/(CAG)₆ double-flap substrate, PCNA did not affect the DNA synthesis activity of pol β at 2 nM (Fig. 7, right panel, compare lanes 12–13 with lane 11). However, it slightly stimulated pol β polymerase activity at 5 nM (Fig. 7, compare lanes 15–16 with lane 14). Thus, the results indicate that PCNA slightly stimulated pol β DNA synthesis during BER in a double-flap intermediate resulting from a CAG repeat hairpin, and therefore led to efficient BER in the hairpin. This further indicates that PCNA stimulation of pol β DNA synthesis activity was mediated by the reannealing of the upstream flap to the template strand, creating more annealed 3'-termini that were extended by pol β . The stimulatory effect was greater for the short double-flaps, supporting the results from Figs. 4 and 5, indicating that PCNA had greater difficulty advancing towards the longer upstream flap, as compared to the shorter flap, mitigating the stimulatory effect during BER in a larger hairpin.

4. Discussion

In this study, we discovered that PCNA promoted CAG repeat deletions and removal of a CAG repeat hairpin (Figs. 1 and 4), thereby preventing CAG repeat expansion during BER in a (CAG)₂₀ duplex or (CAG)₇ and (CAG)₁₄ hairpin. We showed that, in this context, PCNA modulated the stability of the repeats by facilitating the activity of LIG I for ligating a nick in the context of a small template CTG loop (Fig. 3) as well as by stimulating FEN1 flap cleavage activity and pol β DNA synthesis for the resolution of a double-flap intermediate during BER in a CAG repeat hairpin loop (Figs. 5 and 7). We demonstrated that the stimulatory effects of PCNA on prevention of CAG repeat expansion were governed by interactions among the dynamics of CAG repeats and their template, the formation of secondary structures and flaps, the PCNA sliding and processing of repair intermediates by pol β , FEN1 and LIG I (Figs. 2, 3, 5, 6 and 7). Our results support a model by which PCNA prevents TNR expansions by stimulation of TNR deletions and the removal of a TNR hairpin (Fig. 8). When a DNA base lesion occurs in either the duplex region or in the hairpin loop, a DNA glycosylase removes the damaged base leaving an abasic site. For BER of an abasic lesion in a duplex TNR tract, APE1 incises the 5'-side of the abasic site allowing DNA slippage and the formation of a TNR loop that prevents PCNA from sliding toward the downstream to interact with pol β or FEN1 (Fig. 8, the pathway on the left). However, this still allows PCNA to interact with LIG I and stimulate sealing of a nick across a small TNR loop on the template strand facilitating repeat deletion. During BER in a hairpin loop, APE1 5'-incision of the abasic site converts the hairpin into a double-flap intermediate. PCNA sliding pushes the upstream flap to reanneal to the template strand creating a 3'-terminus that can be extended by pol β . The reannealing of the upstream flap to the template further allows PCNA to slide to the downstream to interact with FEN1, thereby stimulating FEN1 cleavage of the downstream flap and ultimately facilitating the removal of the hairpin (Fig. 8, the pathway on the right).

Here, we have identified a new role of PCNA in promoting CAG repeat deletions and the removal of a CAG repeat hairpin to prevent TNR expansions. While PCNA has consistently been shown to stimulate FEN1 on DNA of random sequence [33], it remains unknown

whether the stimulatory effect of PCNA may occur in TNR sequences that can form a variety of secondary structures such as hairpins and loops. In this study, we have shown that in a duplex (CAG)₂₀ that allowed the formation of a CTG repeat loop on the template strand during BER, PCNA failed to stimulate FEN1 activity or pol β activity (Supplementary Figs. S1 and S2). However, we found that PCNA exhibited the stimulatory effects on pol β DNA synthesis activity and FEN1 flap cleavage activity (Figs. 5 and 7) on the double-flap substrates that could not form a loop or hairpin on the template strand (Supplementary Fig. S3). The results indicated that a small template bubble blocked the advance of PCNA along the duplex, preventing it from interacting with FEN1 that was located downstream of the bubble at the flap base. Since PCNA is a homotrimer and as such can potentially bind three interacting proteins, one molecule of PCNA may be sufficient to participate in the repair of one molecule of DNA substrate. However, it is possible that multiple PCNA molecules may slide along one molecule of DNA substrate to act on one DNA repair site.

Interestingly, our results showed that during BER in a TNR hairpin loop, due to the lack of secondary structures in the template strand, PCNA was able to facilitate the reannealing of the upstream strand to the template to stimulate pol β and FEN1 activities, promoting the removal of the TNR hairpin. This further suggests that the domain of PCNA that interacts with the template strand is much more rigid structurally than its domain that interacts with the damaged or upper strand of duplex DNA. This appears to allow the ring shaped protein to readily accommodate a flap structure on the damaged strand rather than a loop or hairpin on the template strand.

Here we found that PCNA stimulation of FEN1 is less when the double flaps are longer. This may be because the double-flap substrates are able to equilibrate to form a variety of intermediates with flaps of varying lengths [12], the longer double-flaps are more likely to retain flaps of a longer length more suitable to stable FEN1 binding than are the shorter flaps. Thus, a shorter flap benefits from PCNA stabilizing FEN1 binding to the substrate, preventing FEN1 from falling off and stimulating its cleavage activity. Furthermore, the stimulation of FEN1 activity by PCNA requires a direct interaction between the two proteins, which can be prevented by an upstream 3'-flap. We found that PCNA sliding along the duplex can facilitate the reannealing of the upstream 3'-flap (Fig. 6). However, a longer flap is more difficult to completely reanneal to the template strand than a short flap, preventing the proteins from interacting as easily to stimulate FEN1 activity (Figs. 4 and 5). Furthermore, longer flaps are capable of forming secondary structures, such as small bubbles, that could block the advance of PCNA along the duplex, preventing its interaction with FEN1, and resulting in less stimulation of repair in the longer double-flaps as compared to the shorter double-flaps (Fig. 4, compare lanes 5–10 with lanes 15–20).

Previous studies have shown that LIG I activity can be stimulated by PCNA in duplex DNA of random sequence [23,40]. Here, we found that PCNA stimulated LIG I activity in a TNR duplex across a small CTG repeat loop on the template strand only in the absence of pol β , but not in the presence of pol β (Figs. 1 and 3). This indicates that PCNA managed to interact with LIG I to stabilize the binding of the enzyme to the nick opposite a small loop on the template strand, stimulating the ligation of the nick. Here we have also shown that a small template bubble forms in the (CAG)₂₀ duplex substrates during BER of an abasic

lesion (Fig. 2). Our results indicate that PCNA is able to interact with and stimulate LIG I across from a template bubble to form a deletion product (Fig. 1 and Fig. 3, left side). However, the stimulation of LIG I activity by PCNA was not observed in the presence of pol β (Fig. 3, right side). This suggests that as pol β synthesizes beyond the template bubble, the nick is translated further downstream beyond the reach of PCNA, preventing interaction with LIG I and stimulation of LIG I activity. An alternate explanation for the lack of LIG I stimulation by PCNA observed in the presence of pol β is that pol β and LIG I compete for binding at the 3'-terminus. However, this may be less likely due to the quick release of the nicked DNA by pol β after synthesis. Our results indicate that PCNA stimulation of LIG I plays an important role in promoting TNR deletion for preventing TNR expansions during BER in a duplex TNR tract.

A direct interaction has been shown between PCNA and pol β [32], although the biological significance of this interaction and/or stimulation of polymerase activity has yet to be identified. Our results showed PCNA stimulation of pol β DNA synthesis activity on the double-flap substrates (Fig. 7). We found that the stimulatory effect was mediated by PCNA sliding that facilitated the reannealing of the upstream flap to the template strand creating more annealed 3'-termini as pol β substrates. This has been supported by the significantly decreased S1 cleavage products on the upstream flaps in the presence of PCNA compared to in the absence of PCNA (Fig. 6). Our results further indicate that PCNA cooperated with pol β functionally to facilitate the processing of a double-intermediate and removal of a hairpin during BER, thereby preventing CAG repeat expansion in this context.

Our study provides evidence of a BER cofactor, PCNA, playing an important role in promoting genome stability by facilitating TNR deletion and hairpin removal in the context of a CAG repeat tract. Previously, we have shown the 3'-5' endo- and exonucleases, such as MUS81/EME1 and APE1, can prevent TNR expansions by promoting TNR hairpin removal [18,19]. This indicates that multiple mechanisms are employed in cells to prevent TNR expansions. Our results further indicate that the coordination among the BER proteins and cofactors APE1, pol β , FEN1, LIG I and PCNA can promote TNR deletion and hairpin removal, and this serves as an efficient mechanism of preventing TNR expansions and shortening expanded TNR tracts. Thus BER enzymes and cofactors can be targeted for the prevention and therapy of TNR expansion-associated neurodegenerative diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.dnarep.2016.10.006>.

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Fig. 1. PCNA stimulates repeat deletion during BER in a duplex (CAG)₂₀ tract. BER reactions were reconstituted with substrates containing a THF residue at either the first (left panel) or tenth (right panel) CAG repeat unit of a (CAG)₂₀ duplex tract in the absence and presence of PCNA. Lanes 1 and 10 indicate the substrate alone. Lanes 2 and 11 correspond to the reactions with substrates precut by APE1 (25 nM). Lanes 3 and 12 correspond to the reactions with the APE1 precut substrates and PCNA (100 nM) alone. Lanes 4–6 and 13–15 correspond to BER reactions reconstituted with APE1 (25 nM), FEN1 (5 nM) and LIG I (1 nM) in the absence and presence of PCNA (50 nM or 100 nM). Lanes 7–9 and 16–18 correspond to BER reconstituted with APE1 (25 nM), FEN1 (5 nM), LIG I (1 nM), and pol β (10 nM) in the absence and presence of PCNA. Substrates were ³²P-labeled at the 3'-end of the damaged strand and are illustrated above each gel.

Fig. 2.

A bubble forms in the template of a duplex (CAG)₂₀ tract during BER. The formation of secondary structures in the template strand of the duplex (CAG)₂₀ substrate with damage at the 5'-end(A) or in the middle (B), was probed using 5U S1 nuclease in the absence (left panel) and presence (right panel) of 100 nM PCNA. (C) The formation of a flap in the upstream strand of the substrate with damage in the middle was also probed with 5U S1 nuclease in the absence (left panel) and presence of PCNA (right panel). Lanes 1 and 8 indicate the substrate alone. Lanes 2 and 9 indicate the substrate precut by APE1. Lanes 3–6 and lanes 10–13 indicate the reactions containing APE1 precut substrate with or without 100 nM PCNA along with 5U S1 nuclease at the time intervals of 1, 5, 10 and 15 min. Lanes 7 and 14 indicate size markers whose length is indicated to the right of the gel. Substrates were ³²P-labeled at the 5'-end of the template strand or 3'-end of the damaged strand as illustrated above each gel. The schematic representation of the loops and flaps formed on the template and damaged strands are illustrated below the gels.

Fig. 3. PCNA stimulates LIG I activity on a nick across from a small loop on the template strand. The effect of PCNA on LIG I activity was determined with the substrate containing a nick across from a small (CTG)₁ loop in the presence of 1 nM and 5 nM LIG I without or with 50 nM and 100 nM PCNA. Lane 1 represents the substrate (25 nM) alone. Lanes 2 and 5 correspond to the reactions containing the substrate and 1 nM and 5 nM LIG I, respectively. Lanes 3–4 and lanes 6–7 correspond to the reactions containing 1 nM or 5 nM LIG I with the presence of 50 nM and 100 nM PCNA. The substrate was ³²P labeled at the 5'-end of the upstream strand of the substrate.

Fig. 4.

PCNA stimulates processing of double-flap intermediates resulting from a CAG repeat hairpin. The effects of PCNA on processing of the double-flap intermediates resulting from a CAG repeat hairpin were determined by reconstituting BER with double-flap substrates containing (CAG)₃/(CAG)₄ flaps (left panel) or (CAG)₆/(CAG)₆ flaps (right panel) that mimic an intermediate generated by APE1 5'-incision in a CAG repeat hairpin loop in the presence and absence of PCNA. Lanes 1 and 11 indicate the size markers of the unexpanded product. Lanes 2 and 12 represent the size markers of the expanded product. Lanes 3 and 13 represent the substrate only. Lanes 4 and 14 correspond to the reactions with the substrates and PCNA (100 nM) only. Lanes 5–7 and lanes 15–17 correspond to BER reactions containing FEN1 (0.5 nM or 1 nM) and LIG I (0.1 nM) in the absence and presence of PCNA (50 nM or 100 nM). Lanes 8–10 and lanes 18–20 correspond to BER reactions containing FEN1 (0.5 nM or 1 nM), LIG I (0.1 nM), and pol β (10 nM) in the absence and presence of PCNA (50 nM or 100 nM). Substrates were ³²P-labeled at the 3'-end of the damage-containing strand and are illustrated above each gel.

Fig. 5. PCNA stimulates FEN1 flap cleavage on double-flap intermediates. The stimulation of FEN1 cleavage by PCNA on double-flap substrates that mimic an intermediate formed during BER in a CAG repeat hairpin was explored by measuring FEN1 activity in the absence and presence of PCNA on substrates containing (CAG)₃/(CAG)₄ flaps (left panel) or (CAG)₆/(CAG)₄ flaps (right panel). Lanes 1 and 10 correspond to the substrate only. Lanes 2 and 11 correspond to reaction mixture containing substrate and PCNA (100 nM) only. Lanes 3–5 and lanes 12–14 correspond to the reactions containing the substrates and FEN1 (0.5 nM or 2.5 nM) in the absence and presence of PCNA (50 nM or 100 nM). Lanes 6 and 15 correspond to reactions containing substrates and pol β (10 nM) in the presence of PCNA (100 nM). Lanes 7–9 and lanes 16–18 correspond to reactions with the substrates, FEN1 (0.5 nM or 2.5 nM) and pol β (10 nM) in the absence and presence of PCNA (50 nM or 100 nM). Substrates were ³²P-labeled at the 3'-end of the damage-containing strand and are illustrated above each gel.

Fig. 6.

The formation of a flap in the upstream strand of double-flap intermediates. (A) The formation of a flap in the upstream strand of the (CAG)₃/(CAG)₄ double-flap substrate was probed using S1nuclease in the absence (left panel) and presence (right panel) of 100 nM PCNA. Lanes 1 and 7 indicate the substrate only. Lanes 2–5 and lanes 8–11 indicate reactions containing substrate with or without 100 nM PCNA incubated with 0.25U S1 nuclease from 1 to 15 min. Lanes 6 and 12 indicate size markers whose length is indicated to the right of the gel. Quantification of the amount of cleavage products is shown below each gel. (B) The formation of a flap in the upstream strand of the (CAG)₆/(CAG)₆ double-flap substrate was probed using S1 nuclease. Lanes 1 and 7 indicate the substrate only. Lanes 2–5 and lanes 8–11 indicate the reactions containing the substrate with or without 100 nM PCNA

incubated with 4U S1 nuclease from 1 to 15 min. Lanes 6 and 12 indicate size markers whose length is indicated to the right of the gel. Quantification of the amount of cleavage products is shown below each gel. Substrates were ^{32}P -labeled at the 5'-end of the upstream strands as illustrated above each gel. The schematic representations of the flaps formed are illustrated below the gels.

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Fig. 7.

PCNA slightly stimulates pol β DNA synthesis on double-flap intermediates during BER. The effect of PCNA on pol β DNA synthesis on double-flap intermediates during BER was measured in the absence and presence of PCNA on substrates containing (CAG)₃/(CAG)₄ (left panel) or (CAG)₆/(CAG)₆ (right panel) double-flaps. Lanes 1 and 9 correspond to the substrate only. Lanes 2 and 10 correspond to the reactions containing the substrates and PCNA (100 nM) only. Lanes 3–5 and lanes 11–13 correspond to reactions containing substrate and pol β (2 nM) without or with PCNA (50 nM or 100 nM). Lanes 6–8 and lanes 14–16 correspond to substrates incubated with pol β (5 nM) without and with PCNA (50 nM or 100 nM). Substrates were ³²P-labeled at the 5'-end of the upstream strand and are illustrated above each gel. The schematic representation is illustrated below the gels.

Fig. 8.

PCNA prevents TNR expansions during BER. Oxidized DNA base lesions induced by DNA damaging agents can occur in a duplex TNR tract (left pathway) or in the loop region of a TNR hairpin (right pathway). During BER within a duplex TNR tract, the formation of a loop structure within the template can block the advance of PCNA toward the downstream, preventing PCNA from interacting with FEN1. PCNA is able to interact with LIG I to stimulate ligation across a small template loop (left pathway). During BER of a lesion located in the loop of a TNR hairpin, the hairpin is converted into a double-flap intermediate. PCNA is then loaded from the upstream and slides toward the downstream pushing the reannealing of the upstream flap to the template strand. This creates a longer 5'-flap for FEN1 to be readily loaded. PCNA then interacts with FEN1 and stimulates FEN1 flap cleavage activity, thereby promoting removal of the hairpin and prevention of TNR expansions.