

R-loops promote trinucleotide repeat deletion through DNA base excision repair enzymatic activities

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Eduardo E. Laverde¹, Yanhao Lai², Fenfei Leng^{1,2,3} , Lata Balakrishnan⁴, Catherine H. Freudenreich⁵ , and Yuan Liu^{1,2,3,*}

From the ¹Biochemistry Ph.D. Program, the ²Department of Chemistry and Biochemistry, and the ³Biomolecular Sciences Institute, Florida International University, Miami, Florida, USA, the ⁴Department of Biology, Indiana Purdue University Indianapolis, Indianapolis, Indiana, USA, and the ⁵Department of Biology, Tufts University, Medford, Massachusetts, USA

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Trinucleotide repeat (TNR) expansion and deletion are responsible for over 40 neurodegenerative diseases and associated with cancer. TNRs can undergo somatic instability that is mediated by DNA damage and repair and gene transcription. Recent studies have pointed toward a role for R-loops in causing TNR expansion and deletion, and it has been shown that base excision repair (BER) can result in CAG repeat deletion from R-loops in yeast. However, it remains unknown how BER in R-loops can mediate TNR instability. In this study, using biochemical approaches, we examined BER enzymatic activities and their influence on TNR R-loops. We found that AP endonuclease 1 incised an abasic site on the nontemplate strand of a TNR R-loop, creating a double-flap intermediate containing an RNA:DNA hybrid that subsequently inhibited polymerase β (pol β) synthesis of TNRs. This stimulated flap endonuclease 1 (FEN1) cleavage of TNRs engaged in an R-loop. Moreover, we showed that FEN1 also efficiently cleaved the RNA strand, facilitating pol β loop/hairpin bypass synthesis and the resolution of TNR R-loops through BER. Consequently, this resulted in fewer TNRs synthesized by pol β than those removed by FEN1, thereby leading to repeat deletion. Our results indicate that TNR R-loops preferentially lead to repeat deletion during BER by disrupting the balance between the addition and removal of TNRs. Our discoveries open a new avenue for the treatment and prevention of repeat expansion diseases and cancer.

Trinucleotide repeat (TNR) instability is responsible for over 40 human neurodegenerative diseases, including Huntington's disease (CAG/CTG) and Friedreich's ataxia (GAA/TTC) among others (1–3) and associated with cancers (4, 5). The molecular basis underlying TNR instability is the formation of non-B-form DNA structures, including hairpins, loops, triplexes, and G-quadruplexes during DNA replication (6), repair (7), recombination (8), and gene transcription (9). TNR tracts are rich in guanines, thus forming hotspots for DNA base lesions, the most common form of DNA damage. It has been proposed that repeated oxidative DNA damage can promote somatic TNR expansions through a “toxic oxidation cycle” (2), presumably through multiple rounds of repeat expansion resulting from the repair of base lesions by DNA base excision repair (BER). Thus, somatic TNR expansion may be essential to

promote TNR length to reach the threshold leading to the evident neurodegenerative symptoms of TNR diseases and governing the age at onset of the diseases. Recent studies from our group and others have shown that BER of a variety of base lesions plays an active role in modulating TNR instability by inducing large repeat deletions and small expansions (10–15). We have further demonstrated that the location of a DNA base lesion in a TNR tract determines the outcome of the repeat deletion or expansion (13).

Gene transcription at expanded repeated sequences can lead to the formation of R-loops (16, 17). R-loops are generated when a nascent RNA strand hybridizes back to its DNA template to create an RNA:DNA hybrid (16, 17). Scheduled R-loops are detected in bacteria, yeast, and humans and are implicated to be involved in many cellular processes (16, 17). Some physiological roles of R-loops include initiation at mitochondrial and prokaryotic origins of DNA replication, class switch recombination at immunoglobulin genes, modulation of DNA methylation at CpGs, and transcription termination. Thus, the formation of scheduled R-loops is essential for cellular function. However, the accumulation of unscheduled R-loops can result in DNA damage and genome instability (16, 17). Defective RNA processing can result in the accumulation of R-loops, leading to activation of DNA damage response (18). Thus, it is suggested that R-loops can act as a mutagenic intermediate disrupting genome integrity.

R-loops can accumulate on expanded TNRs (19–23), and their accumulation can be further promoted by the deficiency of the senataxin helicase that disrupts the RNA:DNA hybrid (24) or depletion of RNase H that removes RNAs (25). Moreover, R-loops on the expanded GAA, CAG, CTG, or CGG repeats result in a guanine-rich single-stranded region on the nontemplate strand, which can be damaged by endogenous and exogenous DNA base-damaging agents. A previous study from the Freudenreich group (23) has shown that the yeast cytosine deaminase, Fcy1, causes R-loop-associated CAG repeat deletions that are dependent on BER. However, the molecular mechanisms by which BER promotes TNR deletion remains unknown.

We hypothesize that BER of DNA base damage in the nontemplate strand of a TNR R-loop results in repeat deletion by promoting the removal of TNRs but preventing the addition of the repeats. To test this, we examined the activities of BER enzymes on TNR R-loops during BER and their impact on the

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* For correspondence: Yuan Liu, yualiu@fiu.edu.

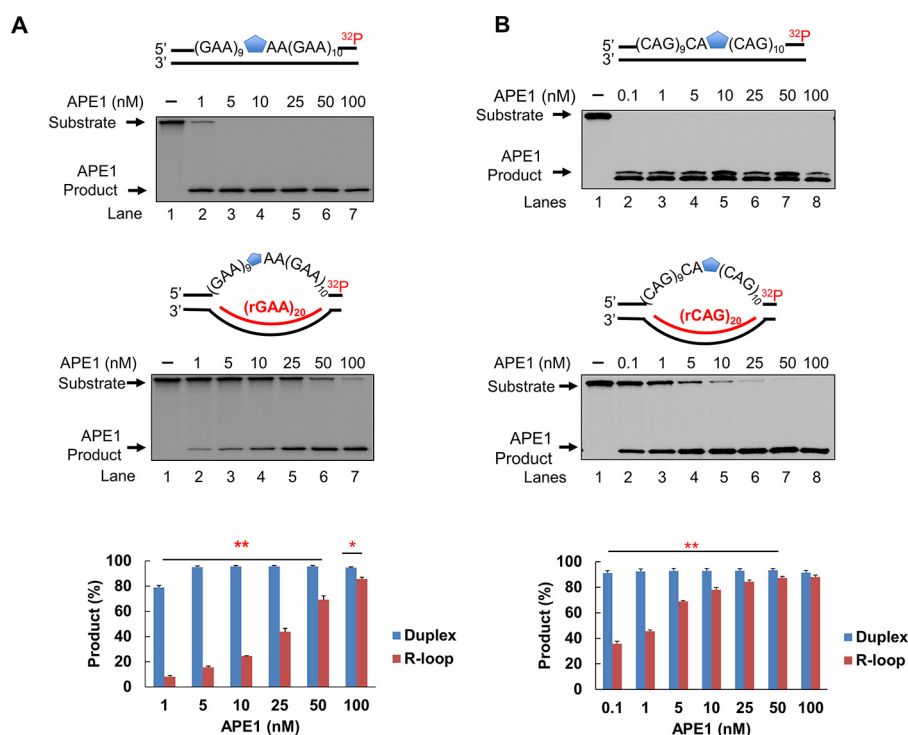


Figure 1. APE1 incision of an abasic site on TNR R-loops. APE1 incision of an abasic site in the middle of the duplex and R-loop substrates containing (GAA)₂₀ and (CAG)₂₀ repeats was performed by incubating various concentrations of APE1 (1–100 nM) with 10 nM substrates at 37 °C for 30 min. Substrates were ³²P-labeled at the 5′-end of the strand containing an abasic site. Substrates were separated from the product in 15% urea-denaturing polyacrylamide gels and were detected by a phosphorimager. Substrates are schematically illustrated above the gels. A, APE1 5′-incision of an abasic site on the duplex DNA substrate containing (GAA)₂₀ or the (GAA)₂₀ repeat R-loop substrate. Lane 1, substrate only; lanes 2–7, APE1 incision activity at concentrations of 1–100 nM. B, APE1 5′-incision of an abasic site on the duplex DNA substrate containing (CAG)₂₀ repeats or the (CAG)₂₀ repeat R-loop substrate. Lane 1, substrate only. Lanes 2–8, reactions with APE1 at concentrations of 0.1–100 nM. The quantification of the APE1 incision product from A and B is shown below the gels. *, significant difference in the products between the duplex DNA and R-loop substrates with *p* < 0.05. **, significant difference with *p* < 0.01. Error bars, S.D.

stability of the repeats. We found that AP endonuclease 1 (APE1) incised an abasic site in (GAA)₂₀ and (CAG)₂₀ repeat R-loops, creating a double-flap intermediate that significantly inhibited DNA polymerase β (pol β) DNA synthesis activity. In contrast, flap endonuclease 1 (FEN1) cleavage of GAA and CAG repeats during BER in TNR R-loops was significantly stimulated. Furthermore, FEN1 also cleaved the RNA strand in TNR R-loops to promote the resolution of the R-loops and repeat deletion. The results indicate that R-loops promote TNR deletion through BER by modulating the activities of BER core enzymes and processing of the RNA strand, thereby altering the balance between the addition and removal of TNRs.

Results

APE1 can incise an abasic site in TNR R-loops during BER

To elucidate the molecular mechanisms by which TNR R-loops induce repeat instability through BER, we initially examined whether APE1 could incise the 5′-end of an abasic site (THF) located in the middle of (GAA)₂₀ and (CAG)₂₀ R-loops (Fig. 1). We found that APE1 (0.1–100 nM) efficiently incised the abasic site located in the duplex (GAA)₂₀ and (CAG)₂₀ repeat substrates (Fig. 1 (A and B), top panels). In contrast, low concentrations of APE1 (0.1–10 nM) exhibited poor 5′-incision of the abasic site in the (GAA)₂₀ and (CAG)₂₀ repeat R-loops (Fig. 1 (A and B), middle panels (lanes 2–4 and 2–5) and bottom panels). However, increasing concentrations from 25 to 100 nM

led to increased incision of the abasic site with all the lesions incised at 100 nM (Fig. 1 (A and B), middle panels (lanes 5–7 and 6–8) and bottom panels). The results showed that the APE1 cleavage activity on the abasic site in the nontemplate single-strand DNA in the (GAA)₂₀ and (CAG)₂₀ R-loops was significantly less efficient compared with its activity in the duplex repeats. However, high concentrations of APE1 at 100 nM incised all the abasic sites in the TNR R-loops. These results are consistent with our previous studies showing that APE1 incision of an abasic site in a single-stranded CAG repeat hairpin was attenuated (15).

The DNA synthesis activity of pol β is inhibited during BER in TNR R-loops

Our previous studies have shown that pol β can bypass loop structures on the TNR template strand to promote repeat deletion on duplex DNA (12, 14). Also, we demonstrated that weak DNA synthesis activity of pol β containing the R137Q polymorphism leads to small CAG repeat deletions (26). Thus, we determined the DNA synthesis activity of pol β at the different concentrations (0.1–50 nM) in the TNR R-loops harboring (rGAA)₂₀/(TTC)₂₀ and (rCAG)₂₀/(CTG)₂₀ repeats with an abasic site in the middle of the nontemplate strand and compared the activity with that from the (GAA)₂₀ and (CAG)₂₀ duplex substrates (Fig. 2). We found that increasing concentrations of pol β (0.1–50 nM) led to a significant increase of pol β DNA

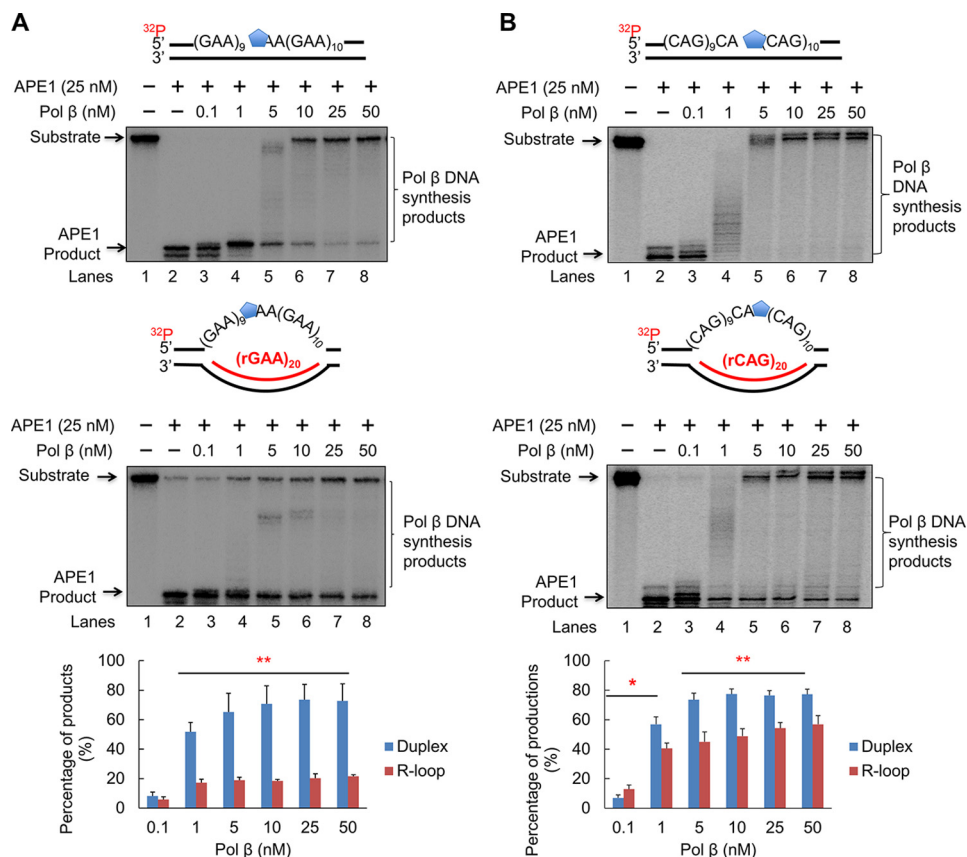


Figure 2. Pol β DNA synthesis on TNR R-loops. Pol β DNA synthesis activity in duplex TNR and R-loop substrates was determined by incubating various concentrations of pol β (0.1–50 nM) with 10 nM duplex DNA or R-loop substrates containing (GAA)₂₀ or (CAG)₂₀ with an abasic site in the middle of the repeats at 37 °C for 30 min. Substrates (10 nM) were ³²P-labeled at the 5'-end of the strand containing an abasic site and incubated with 25 nM APE1 and increasing concentrations of pol β (0.1–50 nM). Substrates and products were separated in a 15% urea-denaturing polyacrylamide gel and detected by a phosphorimager. Substrates are schematically illustrated above the gels. **A**, pol β DNA synthesis on the duplex DNA or R-loop substrate containing (GAA)₂₀ repeats with an abasic lesion in the middle of the repeats. **B**, pol β DNA synthesis on the duplex DNA or R-loop substrate containing (CAG)₂₀ repeats with an abasic site located in the middle of the repeats. Lane 1, substrate only. Lane 2, reaction with 25 nM APE1. Lanes 3–8, reactions with APE1 and different concentrations of pol β (0.1–50 nM). The quantification of the pol β DNA synthesis products is illustrated below the gels. *, significant difference in the APE1 cleavage products between the duplex DNA and R-loop substrates with $p < 0.05$. **, significant difference with $p < 0.01$. Error bars, S.D.

synthesis products (Fig. 2 (A and B), lanes 3–8). However, pol β performed much less DNA synthesis on the TNR R-loop substrates (~20% for the GAA and 40% for CAG repeat R-loop) than it did on the duplex substrates (50–70%) (Fig. 2 (A and B), bar charts below the gels). The results indicate that pol β DNA synthesis on the TNR R-loops was significantly inhibited by the presence of the RNA:DNA hybrid in the R-loops. The results further suggest that the RNA:DNA hybrid displaced the upstream strand of the nontemplate strand of the R-loops into flaps that in turn inhibited pol β DNA synthesis. To confirm this, we examined pol β DNA synthesis activity on nicked (CAG)₂₀ or (GAA)₂₀ duplex and R-loop substrates that harbor a 3'- and 5'-repeat flap on the nontemplate strand with (rGAA)₂₀/(TTC)₂₀ or (rCAG)₂₀/(CTG)₂₀ repeats (Fig. 3). The results showed that pol β (1–50 nM) performed efficient DNA synthesis on the duplex (GAA)₂₀ or (CAG)₂₀ substrates (Fig. 3 (A and B), top panels). However, no pol β DNA synthesis products were detected with the R-loop substrate containing the GAA repeat flaps (Fig. 3A, bottom panel, lanes 2–6), indicating that pol β DNA synthesis was completely inhibited by the presence of the 3'-repeat flap. Pol β performed much less DNA synthesis on nicked (CAG)₂₀ R-loop with the 3'- and 5'-flap than it

did on the duplex repeat substrates (Fig. 3B, bottom panel, lanes 2–6) indicating that the DNA synthesis activity was also significantly inhibited by the 3'-repeat flap displaced by the RNA:DNA hybrid. To further confirm this, we examined the pol β DNA synthesis activity on the R-loop substrates in the presence of *Escherichia coli* RNase HI that removed the RNA strand. We found that the removal of RNA in the TNR R-loops significantly stimulated pol β DNA synthesis activity (Fig. S1), indicating the necessity of the RNA strand for the inhibition of pol β activity. These results indicate that Pol β synthesis activity in the TNR R-loops was significantly inhibited by the formation of a 3'-flap displaced by the RNA strand in the R-loops during BER. It should be noted that some full-length pol β synthesis products shown in Figs. 2 and 3 were detected with the R-loop substrates except the double-flap GAA repeat R-loop substrate. The products appeared to result from the residual duplex DNA. This is supported by the results showing that two-thirds of the GAA and CAG repeat substrates formed R-loops with one-third of the substrates forming duplex DNA (left panels of Fig. S2, A and B). Only 50% of CAG repeats formed double-flap R-loop substrates (right panel of Fig. S2B). Because almost all GAA repeats formed into the double-flap R-loop substrates

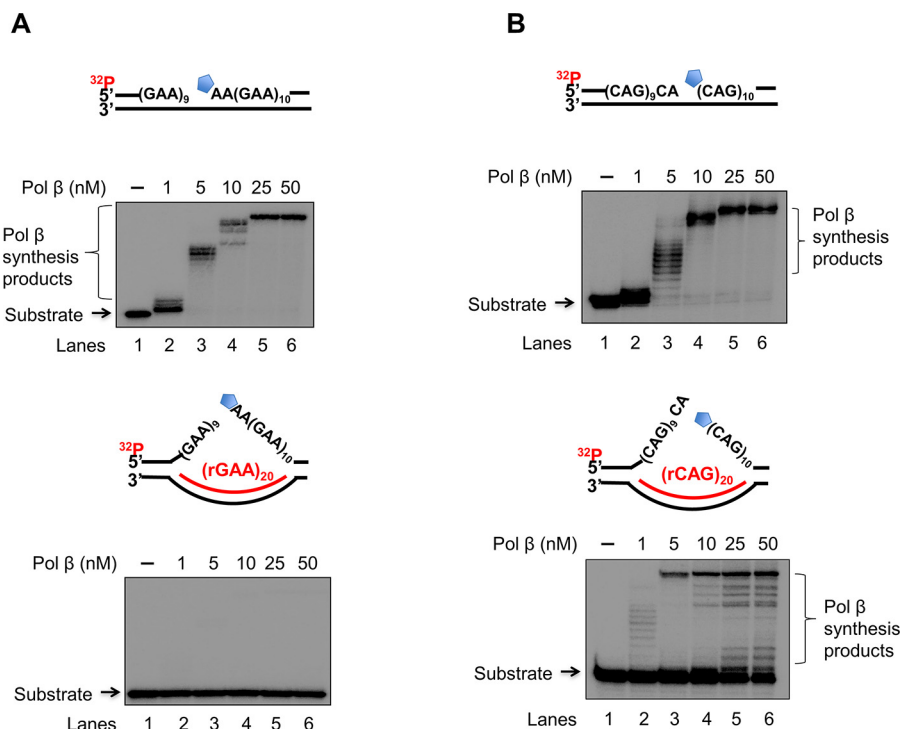


Figure 3. Pol β DNA synthesis on the nicked TNR duplex and nicked R-loops. Pol β DNA synthesis on the nicked duplex or R-loop substrate was determined by incubating the nicked TNR duplex or R-loop substrate with pol β at 1–50 nM at 37 °C for 30 min. Substrates were 32 P-labeled at the 5'-end of the strand containing an abasic site. Substrates and products were separated in a 15% urea-denaturing polyacrylamide gel and detected by a phosphorimager. Substrates are schematically illustrated above the gels. Pol β DNA synthesis products are indicated. **A**, pol β DNA synthesis on the nicked duplex and R-loop substrates containing (GAA)₂₀ with an abasic site in the middle of the repeats. **B**, pol β DNA synthesis on the nicked duplex and R-loop substrates containing (CAG)₂₀. Lane 1, substrate alone. Lanes 2–6, reactions with different concentrations of pol β at 1–50 nM and 25 nM substrates.

(right panel of Fig. S2A), no pol β DNA synthesis products were detected. Thus, the results indicated that the full-length pol β synthesis products were generated from the duplex DNA rather than the R-loop substrates.

FEN1 cleavage of TNRs is stimulated during BER in TNR R-loops

We then reasoned that FEN1 flap cleavage activity of the repeats might be stimulated by the presence of RNA that promoted the formation of a 5'-flap on the nontemplate strand of the R-loop substrates. To test this, we measured the FEN1 cleavage activity on the (GAA)₂₀ and (CAG)₂₀ R-loop substrates containing an abasic site in the middle of the nontemplate strand (Fig. 4) and compared the activity with duplex DNA substrates. We found that increasing concentrations of FEN1 (0.1–50 nM) exhibited weak flap cleavage activity on the TNR duplex substrates with 1–20% cleavage products generated from the (GAA)₂₀ substrate and 1–50% products generated from the (CAG)₂₀ substrate (Fig. 4 (A and B), lanes 3–8, top and bottom panels). However, we found that FEN1 cleavage of the downstream strand on the R-loop substrates at the same concentrations was significantly stimulated with 25–80% products from the (GAA)₂₀ R-loop substrate and 10–60% products from the (CAG)₂₀ R-loop substrate (Fig. 4 (A and B), lanes 3–8 of the middle and bottom panels). This indicated that the RNA:DNA hybrid on the R-loop substrates facilitated the formation of the downstream (GAA)₁₀ and (CAG)₁₀ into a 5'-flap that

was efficiently cleaved by FEN1. We then examined the FEN1 cleavage on the nicked (GAA)₂₀ or (CAG)₂₀ duplex substrate and (GAA)₂₀ or (CAG)₂₀ nicked-R-loop substrate containing a 3'-(GAA)₉ or (CAG)₉ flap and 5'-(GAA)₁₀ or (CAG)₁₀ flap with an (rGAA)₂₀/(TTC)₂₀ or (rCAG)₂₀/(CTG)₂₀ (Fig. 5). We found that FEN1 mainly cleaved one repeat from the (GAA)₂₀ or (CAG)₂₀ nicked-duplex substrate (Fig. 5 (A and B), lanes 2–6 of the top panels). However, FEN1 cleavage on the (GAA)₂₀ R-loop double-flap substrate predominantly generated the (GAA)₁₁ flap cleavage product along with a one-repeat product containing the THF (Fig. 5A, lanes 2–6 of the bottom panel). Similarly, for the nicked (CAG)₂₀ R-loop substrate, FEN1 at all concentrations resulted in the products containing (CAG)₁₀ repeats and (CAG)₁ with the THF (Fig. 5B, lanes 2–6 of the bottom panel). Removal of the RNA strand of the TNR R-loop substrates by bacterial RNase HI resulted in the FEN1 cleavage products containing one repeat with the THF (Fig. S3). The results indicate that FEN1 flap cleavage activity was significantly stimulated in the presence of the RNA:DNA hybrid during BER in the TNR R-loops, and the presence of the RNA strand led to FEN1 cleavage of long TNR flaps and therefore a shift to the removal of longer 5'-flaps.

Pol β DNA synthesis is stimulated in the presence of FEN1 during BER in TNR R-loops

Because pol β can coordinate with FEN1 alternate flap cleavage to promote TNR expansion during BER (7, 27), we

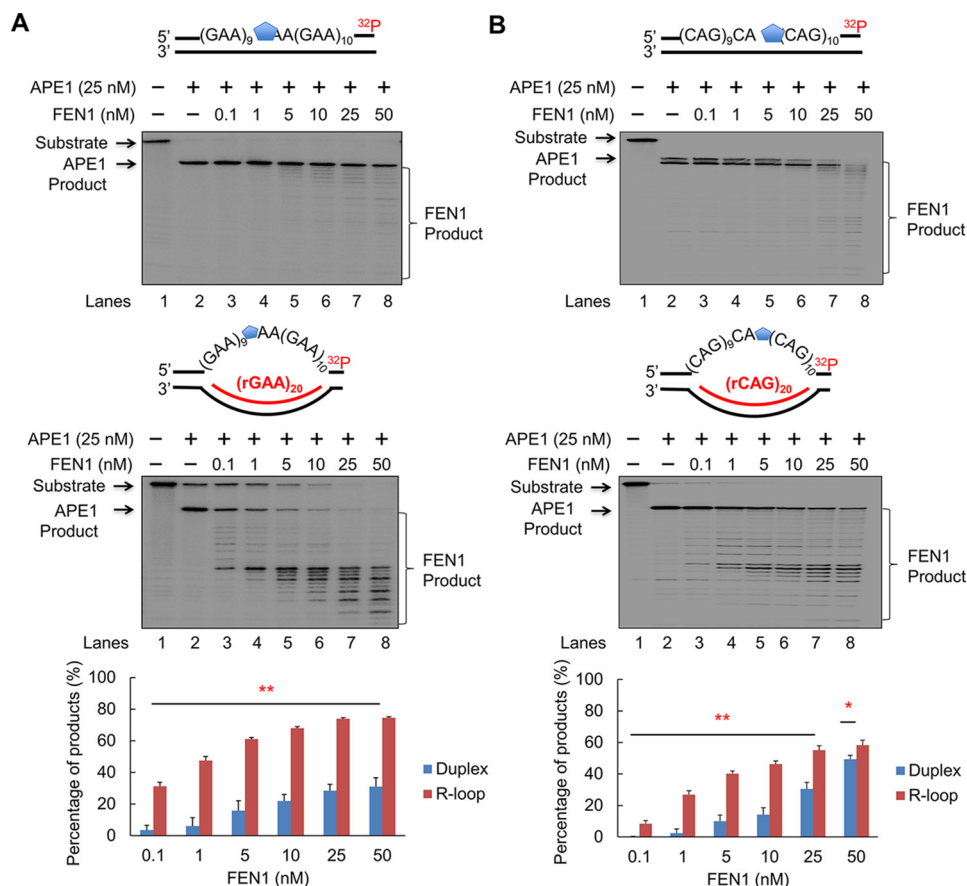


Figure 4. FEN1 cleavage activity on TNR R-loops. FEN1 cleavage of TNRs during BER in duplex TNRs or R-loops was determined by incubating the (GAA)₂₀ or (CAG)₂₀ repeat duplex and R-loop substrate with various concentrations of FEN1 (0.1–25 nM) at 37 °C for 30 min. Substrates were ³²P-labeled at the 3′-end of the strand containing an abasic site and are illustrated above the gels. Substrates and products were separated using a 15% urea-denaturing polyacrylamide gel and detected by a phosphorimager. The quantification of FEN1 cleavage products is shown in the bar chart below the gels. A, FEN1 cleavage activity on duplex or R-loop substrate containing (GAA)₂₀ with an abasic site in the middle of the repeats. B, FEN1 cleavage on the duplex or R-loop substrate containing (CAG)₂₀ with an abasic site in the middle of the repeats. Lane 1, substrate only. Lane 2, reaction with 25 nM APE1. Lanes 3–8, reactions with 0.1–50 nM FEN1 in the presence of 25 nM APE1. *, significant difference in the FEN1 cleavage products between the duplex and R-loop substrate with *p* < 0.05. **, significance with *p* < 0.01. Error bars, S.D.

examined whether pol β and FEN1 could coordinate to promote TNR deletion during BER in the (GAA)₂₀ and (CAG)₂₀ R-loop substrates (Fig. 6). We found that for the nicked (GAA)₂₀ repeat R-loop substrate, pol β did not perform DNA synthesis in the absence or the presence of a low concentration of FEN1 (1 nM) (Fig. 6A, lanes 2 and 3). However, with increasing concentrations of FEN1 from 5 to 25 nM, pol β was able to synthesize GAA repeats, and the synthesis products were significantly increased (Fig. 6A, lanes 4–6). Similarly, for the nicked (CAG)₂₀ repeat R-loop substrate, pol β DNA synthesis products were detected in the presence of 10 and 25 nM FEN1 but not 1 and 5 nM FEN1 (Fig. 6B, compare lanes 5 and 6 with lanes 3 and 4). It should also be noted that the full-length pol β DNA synthesis products were detected with the CAG repeat double-flap R-loop substrate in the absence and presence of FEN1 (Fig. 6B). However, the amount of the products was not altered by the presence of FEN1, indicating that the production of the pol β full-length synthesis products was independent of FEN1 cleavage. This further suggests that the pol β products resulted from the residual duplex DNA rather than from the R-loop substrates. This notion is also supported by the fact that only half of CAG repeats formed the double-flap R-loop substrate (Fig.

S2B). Thus, our results indicate that the pol β synthesis of the repeat-containing R-loop substrates was specifically stimulated by FEN1 flap cleavage. We then tested the effects of pol β DNA synthesis on the FEN1 flap cleavage activity on the R-loop substrates. The results showed that pol β at 1–10 nM stimulated the FEN1 cleavage of the downstream 5′-GAA and -CAG repeat flaps (Fig. 6 (C and D), lanes 3–5), indicating that pol β DNA synthesis strand-displaced the downstream strand facilitating the FEN1 cleavage of a long repeat flap. However, high concentrations of pol β (25–50 nM) inhibited FEN1 cleavage of a long flap and promoted FEN1 alternate flap cleavage of a short repeat flap (Fig. 6 (C and D), lanes 6 and 7). This suggests that the high concentrations of pol β displaced the RNA strand of the TNR R-loops, preventing FEN1 from binding to the DNA flaps and their cleavage. We further tested this possibility by preincubating various concentrations of pol β at 5–50 nM with the GAA and CAG repeat R-loop substrates in the absence of FEN1 (Fig. S4). This allowed pol β to perform the strand displacement synthesis to displace the RNA strand generating an RNA flap. We then used FEN1 to detect the displaced RNA flap by pol β DNA synthesis. The results showed that with increasing concentrations of pol β (5–50 nM), FEN1 cleavage on CAG

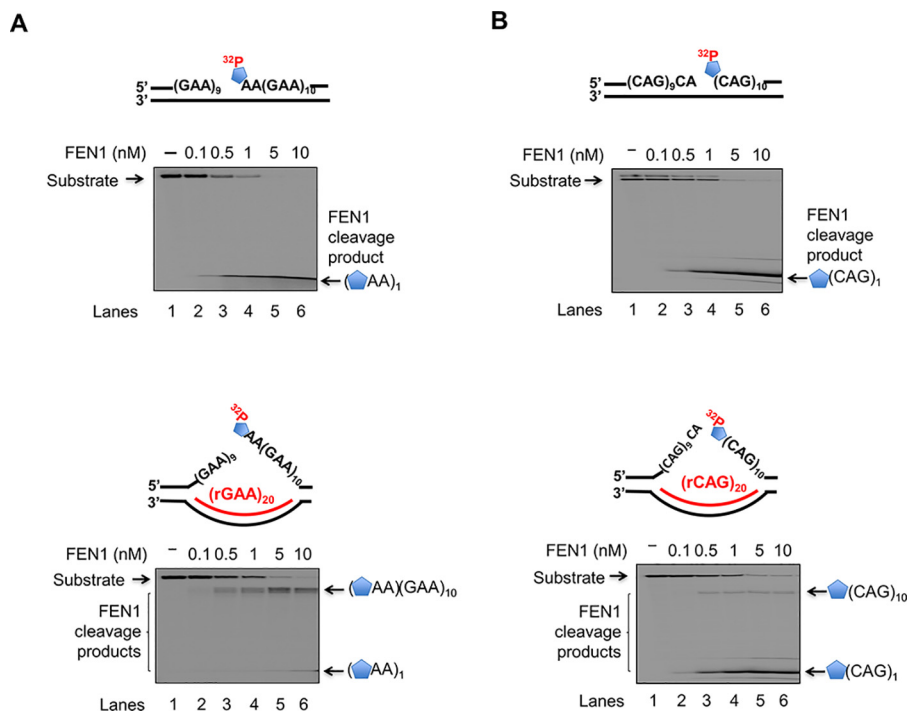


Figure 5. FEN1 cleavage activity on the nicked TNR R-loops. FEN1 cleavage of TNRs on the nicked duplex and R-loop substrates was examined by incubating the nicked duplex or R-loop substrates (10 nM) containing (GAA)₂₀ or (CAG)₂₀ repeats with FEN1 (0.1–25 nM) at 37 °C for 30 min. Substrates were ³²P-labeled at the 5′-end of the downstream strand and illustrated above the gels. Substrates and products were separated using a 15% urea-denaturing polyacrylamide gel and detected by a phosphorimager. FEN1 cleavage products are indicated. **A**, FEN1 cleavage activity on the duplex (the gel on the top) and double-flap R-loop substrate containing (GAA)₂₀ and an abasic site in the middle of the repeats (the gel on the bottom). **B**, FEN1 cleavage of CAG repeats on the duplex substrate (the gel on the top) or R-loop substrate (the gel at the bottom) containing (CAG)₂₀ with an abasic site in the middle of the repeats. Lane 1, substrate only. Lanes 2–7, reactions with different concentrations of FEN1 (0.1–25 nM).

and GAA repeat R-loop substrates resulted in a significant increase of the larger size of cleavage products compared with those in the absence of pol β (Fig. S4, compare lanes 3–5 with lane 2). This indicated that high concentrations of pol β performed strand displacement synthesis to displace the RNA strand that interfered with FEN1 cleavage of the flap on the non-template strand.

FEN1 cleaves the RNA strand in TNR R-loops during BER

Because we found that FEN1 efficiently cleaved the DNA strand in an R-loop, we further tested whether FEN1 could also cleave the RNA from the RNA:DNA hybrid in the TNR R-loop substrates (Fig. 7). Surprisingly, we found that FEN1 at 0.1–10 nM efficiently cleaved the RNA strand of the nicked (GAA)₂₀ and (CAG)₂₀ repeat R-loop substrates (Fig. 7, A (lanes 2–6) and B (lanes 2–5)). We then examined whether FEN1 cleaved the RNA strand using the same catalytic site as the one for its cleavage of a DNA flap. Employing the FEN1 endonuclease-dead mutant protein, FEN1D181A (28), we showed that the FEN1D181A mutant protein failed to generate any cleavage products on the RNA of the R-loop substrates (Fig. S5), indicating that FEN1 cleaved the RNA strand in the R-loops using the same catalytic site as its DNA endonucleolytic flap cleavage. We further demonstrated that the FEN1 cleavage pattern and activity on the RNA strand was not altered by pol β DNA synthesis (Fig. 7, A (lanes 8–12) and B (lanes 7–10)). The results showed that FEN1 cleaved the RNA strand in the TNR R-loop

substrates independent of pol β DNA synthesis during BER, suggesting that pol β at 5 nM failed to perform DNA synthesis to displace the RNA strand in the presence of FEN1. These results indicate that FEN1 removed the downstream 5′-repeat flap and RNA strand of a TNR-R-loop before pol β could synthesize the repeats. Subsequently, this allowed the reannealing of the upstream strand to the template strand that facilitated pol β DNA synthesis during BER.

BER on TNR R-loops promotes repeat deletion

To further examine whether the weak synthesis of TNRs by pol β and efficient removal of the repeats by FEN1 during BER in a TNR R-loop could promote repeat deletion, we determined TNR instability resulting from BER in a TNR R-loop (Fig. 8). We found that BER in the (GAA)₂₀ and (CAG)₂₀ repeat R-loop substrates resulted in repaired products containing both full-length and deletion products (Fig. S6). Further analysis of the size of the repaired products using capillary electrophoresis and DNA fragment analysis showed that BER led to the products with both full-length and shorter repeats (Fig. 8). For the GAA repeat R-loop substrate, BER resulted in a large amount of the deletion products containing (GAA)₁₈ and (GAA)_{7–14} repeats (Fig. 8A). For the CAG repeat R-loop substrate, BER resulted in deletion products containing (CAG)_{18–19} and (CAG)_{7–14} (Fig. 8B). However, BER of an abasic site in the middle of duplex GAA and CAG repeats only resulted in a small amount of deletion products containing (GAA)_{18–19} and (CAG)_{18–19} repeats (Fig. 8, A and B).

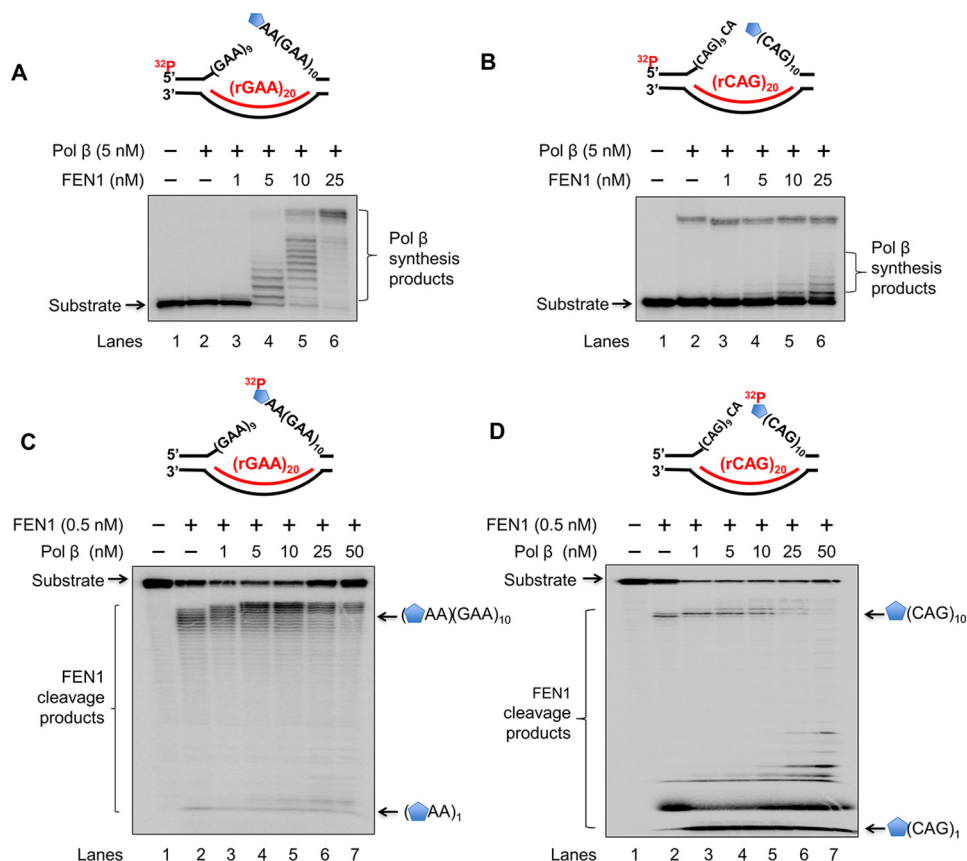


Figure 6. Coordination of FEN1 flap cleavage and pol β DNA synthesis in TNR R-loops during BER. The coordination between FEN1 cleavage of TNRs and pol β synthesis of the repeats in R-loops was determined by testing pol β DNA synthesis in the presence of various concentrations of FEN1 or by examining FEN1 cleavage activity with the presence of different concentrations of pol β . Substrates (10 nM) were incubated with 5 nM pol β and different concentrations of FEN1 (1–25 nM) (A and B) or 0.5 nM FEN1 and increasing concentrations of pol β (1–50 nM) (C and D) at 37 °C for 30 min. A and B, pol β DNA synthesis activity in the presence of FEN1 at concentrations of 1–25 nM with the nicked (GAA)₂₀ and (CAG)₂₀ R-loop double-flap substrates. Substrates were ³²P-labeled at the 5′-end of the upstream strand. Lane 1, substrate alone. Lane 2, reaction with 5 nM pol β . Lanes 3–6, reactions with 5 nM pol β in the presence of various concentrations of FEN1 at 1–25 nM. C and D, FEN1 cleavage of TNRs in the presence of various concentrations of pol β (1–50 nM) with the nicked (GAA)₂₀ and (CAG)₂₀ repeat R-loop substrates containing the double-flaps. Lane 1, substrate alone. Lane 2, reaction with 0.5 nM FEN1. Lanes 3–6, reactions with 0.5 nM FEN1 in the presence of different concentrations of pol β (1–50 nM). Substrates were ³²P-labeled at the 5′-end of the downstream strand and illustrated above the gels. Substrates and products were separated in a 15% urea-denaturing polyacrylamide gel and detected by a phosphorimager. Pol β DNA synthesis products and FEN1 cleavage products are indicated.

These results indicate that TNR R-loops promoted TNR deletions through BER and shifted the most common size, ranging from small deletions of 1–2 repeats to larger deletions of 6–13 repeats.

Discussion

In this study, we explored the underlying mechanisms of R-loop-induced repeat instability by determining the activities of BER enzymes and their coordination during BER in TNR R-loops. We demonstrated that an abasic site in the nontemplate strand of (CAG)₂₀ and (GAA)₂₀ repeat R-loops was incised by APE1, resulting in a double-flap intermediate with a 5′- and 3′-flap containing an RNA:DNA hybrid (Fig. 1). We found that pol β synthesis of repeats on the R-loops was significantly inhibited (Figs. 2 and 3). In contrast, FEN1 flap cleavage of the 5′-flap was significantly stimulated and biased more toward cleavage at the end of the hybrid, resulting in the release of a large flap (Figs. 4 and 5). We found that FEN1 cleavage facilitated pol β synthesis of TNRs (Fig. 6, A and B). However,

the presence of pol β suppressed FEN1 cleavage of the repeats (Fig. 6, C and D). We further demonstrated that FEN1 efficiently cleaved the RNA strand of the TNR R-loops during BER, and this was not affected by pol β (Fig. 7), suggesting that pol β failed to displace the RNA strand to create an RNA flap on the R-loop in the presence of FEN1 and that FEN1 cleavage of the RNA is what allowed the limited pol β synthesis. Finally, we found that BER in the R-loops led to an increase in TNR deletions and a shift to larger deletions compared with repair in a duplex substrate (Fig. 8). All the results support a hypothetical model for the repair of abasic lesions generated from a DNA base lesion in the nontemplate strand of a TNR R-loop (Fig. 9). APE1 incises the abasic site, resulting in a single-strand break and the formation of a double-flap intermediate with an RNA:DNA hybrid on the template strand, along with an upstream 3′-flap and downstream 5′-flap. The 3′-flap inhibits pol β synthesis of TNRs. The 5′-flap stimulates FEN1 cleavage on the downstream strand. Subsequently, FEN1 cleaves the RNA strand in the TNR R-loops, leading to the dissociation of the RNA from the R-loops and facilitating pol β bypassing of

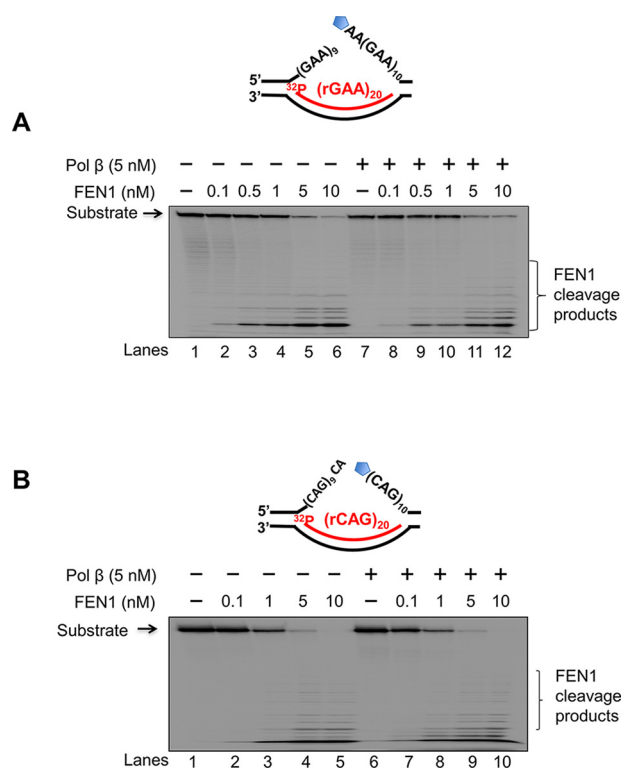


Figure 7. FEN1 cleaves the RNA strand of TNR R-loops. The cleavage of the RNA strand of TNR R-loops by FEN1 was determined using the nicked-R-loop substrates containing an (rGAA)₂₀/(TTC)₂₀ (A) or (rCAG)₂₀/(CTG)₂₀ (B) RNA:DNA hybrid. Substrates were ³²P-labeled at the 5'-end of the RNA strand in the R-loop substrates, schematically illustrated above the gels. Substrates (10 nM) were incubated with FEN1 in the absence or presence of pol β at 37 °C for 30 min. Substrates and products were separated in a 15% urea-denaturing polyacrylamide gel and detected by a phosphorimager. A, FEN1 cleavage of the (rGAA)₂₀ repeats in the GAA repeat nicked-R-loop substrate in the absence or presence of 5 nM pol β. Lane 1, substrate only. Lanes 2–6, reactions with various concentrations of FEN1 at 0.1–10 nM without pol β. Lanes 7–12, reactions with FEN1 at 0.1–10 nM in the presence of 5 nM pol β. B, FEN1 cleavage of the (rCAG)₂₀ repeats in the CAG repeat nicked-R-loop double-flap substrate without or with 5 nM pol β. Lane 1, substrate only. Lanes 2–5, reactions with various concentrations of FEN1 at 0.1–10 nM without pol β. Lanes 7–10, reactions with FEN1 at 0.1–10 nM in the presence of 5 nM pol β.

secondary structures, such as a loop or hairpin, to perform DNA synthesis. This results in more repeats removed than synthesized during BER in R-loops, promoting large repeat deletions (Fig. 9). It should be noted that in cells, expanded GAA repeats can also form a more complex triplex structure compared with the hairpin structures formed in the expanded CAG repeats. This may modulate the processes by which the repeats can be deleted through BER. Furthermore, the loop and hairpin structures formed on the template strand of GAA and CAG repeats can also allow replication DNA polymerases to skip over the structures during DNA replication, thereby promoting repeat deletion (Fig. 9).

The role of R-loops in modulating TNR instability has been implicated by several studies (19, 20). Loomis *et al.* (22) have found that hairpin structures can form in the single-stranded nontemplate strand of a CGG repeat R-loop, suggesting that these secondary structures may underlie repeat expansions. Neil *et al.* (29) demonstrate that R-loops formed on the long GAA repeats can result in the formation of triplex structures, causing repeat expansion during DNA replication. A recent

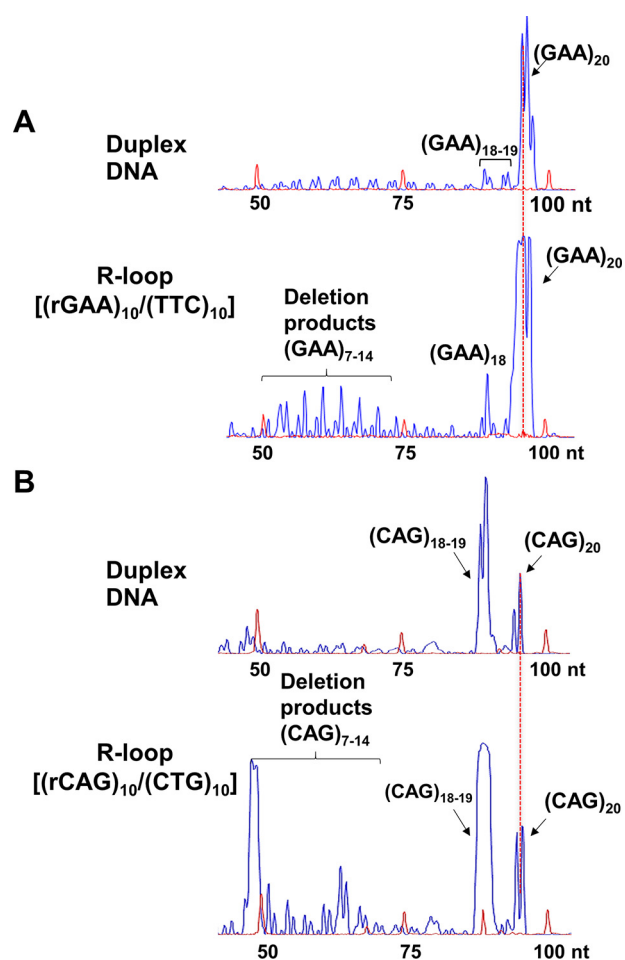


Figure 8. BER in R-loops promotes TNR deletion. BER in TNR R-loops was reconstituted by incubating 25 nM APE1, 5 nM pol β, 10 nM FEN1, and 10 nM LIG I with 25 nM (GAA)₂₀ and (CAG)₂₀ repeat duplex or R-loop substrates containing an abasic site at 37 °C for 30 min. The repair products were isolated and amplified by PCR. The PCR-amplified repaired products were subject to capillary electrophoresis, and repeat sizes were determined by DNA fragment analysis via GeneMapper version 5. A, BER in the (GAA)₂₀ duplex DNA substrate with an abasic site led to the production of a small amount of repeat deletion products containing (GAA)₁₈₋₁₉ repeats. BER of an abasic site in the (GAA)₂₀ R-loop substrate resulted in the production of repeat deletion products of (GAA)₇₋₁₄ and (GAA)₁₈. B, BER was reconstituted with the (CAG)₂₀ repeat duplex or R-loop substrate with an abasic site in the middle of the repeats. The repair products were isolated and amplified by PCR.

study from the Freudenreich group has demonstrated that R-loops formed in CAG repeats result in repeat deletions through DNA base damage and the BER pathway (23). In this study, we demonstrated that BER of DNA base lesions in the nontemplate strand of a TNR R-loop disrupted the balance between the synthesis of TNRs by pol β and their removal by FEN1 via the inhibition of pol β DNA synthesis (Fig. 3) and stimulation of FEN1 large flap cleavage activity (Fig. 4). We showed that the RNA strand of the R-loops played a crucial role in modulating the activities of these BER enzymes (Figs. S1 and S3) by displacing the up- and downstream strands to create the 3'- and 5'-flaps, thereby inhibiting pol β DNA synthesis and stimulating FEN1 flap cleavage during BER. We propose that this situation results in more TNR deletions either by preventing the option of expansions via incorporation of unprocessed

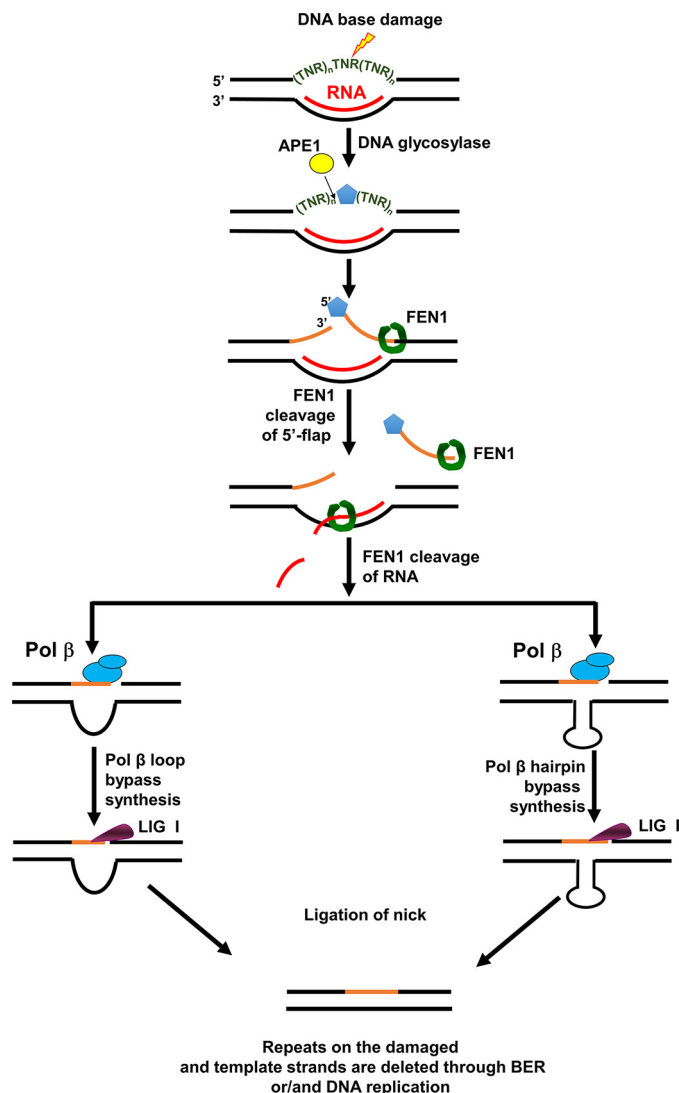


Figure 9. BER in R-loops leads to TNR deletion. DNA base damage that is induced in the nontemplate strand of TNR R-loops is removed by a DNA glycosylase, leaving an abasic site that is incised by APE1 at the 5'-end. Subsequently, this results in a nick and the formation of a double-flap intermediate with an upstream 3'-flap and downstream 5'-flap stabilized by the RNA:DNA hybrid in the R-loop. FEN1 efficiently cleaves the 5'-flap, whereas pol β DNA synthesis is inhibited by the 3'-flap. Subsequently, FEN1 cleaves the RNA strand, leaving a short segment of RNA that dissociates from the template. This results in the formation of secondary structures, such as a loop, hairpin, or triplex structure, in the template strand. Pol β performs bypassing synthesis to skip over the template loop, hairpin, or triplex structure, generating a ligatable nick that is sealed by LIG I. Consequently, this results in more repeats removed by FEN1 than those synthesized by pol β , thereby promoting repeat deletion. The secondary structures on the template strand can also be bypassed by replication DNA polymerases, resulting in repeat deletion during DNA replication.

flaps or by increasing the possibility of pol β hairpin/loop bypass synthesis (14).

Here, we identified a unique mechanism that promotes TNR deletion via BER in R-loops through the stimulation of FEN1 cleavage of TNRs during BER in R-loops (Fig. 9). However, we cannot exclude the possibility that BER cofactors not studied here also play a role. For example, proliferating cell nuclear antigen that can stimulate FEN1 cleavage on TNR flaps (10) may also facilitate FEN1 cleavage of TNR flaps in R-loops, thereby further promoting repeat deletion through BER. More-

over, it is possible that other 5'-endonucleases, exonuclease I (30) and Fanconi anemia-associated nuclease 1 (FAN1) (31) may coordinate with FEN1 to remove the 5'-TNR flap formed on the nontemplate strand of TNR R-loops during BER. This is supported by the fact that both exonuclease I and FAN1 can prevent TNR expansions in mice (32, 33), and FAN1 human polymorphic variations are associated with a late age of onset of several CAG repeat expansion neurodegenerative diseases (34–36). The synergistic effects of the 5'-endo and exonucleases on the processing of TNR R-loops need to be elucidated in the future.

Our study also suggests that BER-mediated TNR deletion via R-loops can serve as a new pathway that resolves R-loops formed in TNRs, attenuating TNR expansion and preventing R-loop-induced gene silencing in the expanded TNRs. It has been shown that R-loops are generated during gene transcription in open chromatin and can inhibit DNA methyltransferase I activity, ensuring the sustainment of an open conformation of chromatin during gene transcription (37). However, it is also found that R-loops formed on the expanded TNRs promote heterochromatinization via the recruitment of G9a methyltransferase and increase H3K9me2 on the repeats, inducing gene silencing (38). This suggests that R-loops on TNRs, if not resolved, can ultimately lead to heterochromatinization and gene silencing. Thus, BER-mediated resolution of TNR R-loops and TNR deletions may be an important protection against the development of TNR diseases. This pathway could potentially be exploited as a new therapy for TNR expansion diseases by targeting expanded TNRs and their associated heterochromatinization and gene silencing.

Experimental Procedures

Materials

The DNA and RNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The dNTPs were purchased from Sigma-Aldrich. The radionucleotides [α - 32 P]cordycepin 5-triphosphate (5000 Ci/mmol) and [γ - 32 P]ATP (3000 Ci/mmol) were purchased from PerkinElmer Life Sciences. Micro Bio-Spin 6 chromatography columns were purchased from Bio-Rad. Terminal deoxynucleotidyl transferase and T4 polynucleotide kinase were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Diethyl pyrocarbonate was purchased from MP Biomedicals (Santa Ana, CA, USA). All chemical reagents were purchased from Thermo Fisher Scientific and Sigma-Aldrich. *E. coli* RNase HI was expressed and purified according to a procedure described previously (39).

Purification of BER enzymes

Recombinant human APE1, FEN1, FEN1D181A mutant protein, and DNA ligase I (LIG I) were expressed and purified as described previously (10, 11). Human recombinant pol β -His₆ tag was expressed and purified as reported previously (26) with minor modifications. Briefly, pol β expression in 2 liters of *E. coli* bacterial cell BL21(DE)3 culture was induced at an optical density of 0.6 at 37°C with 1 mM isopropyl β -D-1-

thiogalactopyranoside (VWR International, Radnor, PA) for 3.5 h. Bacterial cells were pelleted and resuspended with Buffer 1 containing 30 mM HEPES, pH 8.0, 500 mM NaCl, 30 mM imidazole, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and one protease inhibitor mixture tablet (Roche Applied Science). Bacterial cells were lysed by a French Press (Glen Mills, Clifton, NJ). The soluble fraction of the cell lysates, the supernatant, was collected by centrifugation at 12,000 rpm for 30 min. The supernatant was loaded into a nickel-nitrilotriacetic acid–agarose column (Qiagen, Hilden, Germany), followed by a wash with 5 column volumes of Buffer 2, containing 30 mM HEPES, pH 8.0, 1 M NaCl, 30 mM imidazole, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and one protease inhibitor mixture tablet (Roche Applied Science) and then by 5 column volumes of Buffer 1. Pol β was eluted by an imidazole gradient from 30 to 600 mM with Buffer 3, containing 30 mM HEPES, pH 8.0, 500 mM NaCl, 600 mM imidazole, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. The peak fractions were combined and dialyzed into 30 mM HEPES, pH 7.5, 1.7 M $(\text{NH}_4)_2\text{SO}_4$, 0.5% inositol, and 1 mM PMSF. The dialyzed samples were loaded into a phenyl-Sepharose 6 fast flow column (GE Healthcare Bio-Science, Uppsala, Sweden) and eluted with a 30 mM $(\text{NH}_4)_2\text{SO}_4$ reverse gradient. The fractions were tested for enzymatic activity and contamination of *E. coli* DNA polymerases and nucleases. The fractions with a high level of enzymatic activity and low level of polymerase and nuclease contamination were combined and dialyzed into the buffer, containing 30 mM HEPES, pH 7.5, 30 mM KCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF. The dialyzed proteins were loaded on a Mono Q column (GE Healthcare Bio-Science) and eluted by a 1 M KCl gradient. The peak protein fractions with the high level of enzymatic activity with no polymerase and nuclease contamination were combined and dialyzed into the storage buffer containing 30 mM HEPES, pH 7.5, 50 mM KCl, 20% glycerol, and 1 mM PMSF. The fractions were aliquoted and stored at -80°C .

Construction of R-loop oligonucleotide substrates

DNA oligonucleotide substrates containing a THF residue, an analog of an abasic site, were designed to mimic a scenario where an abasic lesion occurs in the middle of a $(\text{GAA})_{20}$ and $(\text{CAG})_{20}$ repeat tract. The guanine at the 10th unit of $(\text{GAA})_{20}$ or $(\text{CAG})_{20}$ repeats was substituted with the THF residue. The sequences of the oligonucleotides are listed in Table S1. The $(\text{GAA})_{20}$ and $(\text{CAG})_{20}$ duplex substrates were constructed by annealing the THF-containing oligonucleotide to its template strand at a molar ratio of 1:3. The $(\text{GAA})_{20}$ and $(\text{CAG})_{20}$ R-loop substrates were constructed by annealing the damage-containing strand, the template strand, and the RNA strand containing $(\text{rGAA})_{20}$ or $(\text{rCAG})_{20}$ at a molar ratio of 1:3:15. The $(\text{GAA})_{20}$ and $(\text{CAG})_{20}$ substrates containing a nick were constructed by annealing the upstream and downstream primer with the template strand at a molar ratio of 1:3:5. The double-flap $(\text{GAA})_{20}$ and $(\text{CAG})_{20}$ substrates with an RNA:DNA hybrid that mimic nicked-R-loop intermediates were constructed by annealing the upstream primer, downstream primer, and the RNA strands with $(\text{rGAA})_{20}$ or $(\text{rCAG})_{20}$ at a ratio of 1:3:15:5 in a

total of 10 μl of annealing reaction. Reagents and buffers used for constructing R-loop substrates were prepared with 0.1% diethyl pyrocarbonate–treated water. The duplex and nicked-duplex substrates were constructed by denaturation at 95°C for 10 min and subsequent cooling down to room temperature. The R-loop substrates and nicked-R-loop substrates were constructed by denaturation at 96°C for 10 min, cooling down to 52°C , and then immediately freezing on dry ice with 100% ethanol. The R-loop substrates constructed were verified using 8% native polyacrylamide gel (Fig. S2)

Enzymatic assay and repeat size measurement

BER reactions were performed by incubating various types of oligonucleotide substrates with purified APE1, pol β , FEN1, or FEN1D181A in BER reaction buffer containing 30 mM HEPES (pH 7.5), 50 mM KCl, 0.1 mg/ml BSA, 0.1 mM EDTA, and 0.01% Nonidet P-40. BER reconstitution reactions with TNR-R-loop and TNR-duplex containing an abasic site were performed by incubating 50 nM APE1, 10 nM pol β , 10 nM FEN1, and 20 nM LIG I, with 5 nM $(\text{GAA})_{20}$ or $(\text{CAG})_{20}$ repeat-containing substrates. All reaction mixtures (20 μl) were assembled on ice in 1 $\times$ BER reaction buffer in the presence of 50 μM dNTPs, 5 mM Mg^{2+} , 2 mM ATP, and the indicated concentrations of BER enzymes and substrates. The reaction mixtures were incubated at 37°C for 30 min. Subsequently, the reaction mixtures were stopped with a 2 $\times$ stopping buffer containing 95% deionized formamide and 10 mM EDTA. To determine the repeat size changes during BER of an abasic site, repaired products were isolated and amplified by PCR. The amplified repaired products were separated by capillary electrophoresis. The sizes of GAA and CAG repeats were determined by DNA fragment analysis according to the method described previously (26).

Data availability

All data described are contained within the article.

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R-loops promote trinucleotide repeat deletion through BER

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: TNR, trinucleotide repeat; BER, base excision repair; pol β , polymerase β ; FEN1, flap endonuclease 1; FAN1, Fanconi anemia-associated nuclease 1; LIG I, DNA ligase I; PMSF, phenylmethylsulfonyl fluoride.

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