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Methods to Study Trinucleotide Repeat Instability Induced by DNA Damage and Repair

Yanhao Lai^{#1}, Ruipeng Lei^{#2}, Yaou Ren², Yuan Liu^{3,4,5}

¹Department of Chemistry and Biochemistry, Florida International University, Miami, FL, USA.

²Biochemistry Ph.D. Program, Florida International University, Miami, FL, USA.

³Department of Chemistry and Biochemistry, Florida International University, Miami, FL, USA.

⁴Biochemistry Ph.D. Program, Florida International University, Miami, FL, USA.

⁵Biomolecular Sciences Institute, Florida International University, Miami, FL, USA.

These authors contributed equally to this work.

Abstract

Trinucleotide repeat (TNR) instability (expansion and deletion) is associated with more than 42 human neurodegenerative diseases and cancer and mediated by DNA replication, repair, recombination, and gene transcription. Somatic TNR instability is involved in the progression of TNR expansion diseases and can be modulated by DNA damage repair and gene transcription. Recent studies from our group and others have shown that DNA base damage and its repair play an active role in modulating TNR instability and are responsible for somatic age-dependent CAG repeat expansion in neurons of Huntington's disease mice induced by oxidative DNA damage. However, it remains to be elucidated how DNA damage, non-B form DNA structures, and DNA repair enzymes and cofactors can coordinate to regulate somatic TNR instability. Understanding the molecular mechanisms underlying DNA damage and repair-mediated somatic TNR instability is critically important for identification of new therapeutic targets for treatment and prevention of TNR-related diseases. Here we describe the methods to study the locations and distribution of DNA base lesions and their effects on TNR instability through DNA base excision repair in in vitro reconstituted human systems.

Keywords

Trinucleotide repeat instability; DNA base excision repair; DNA damage in repeated sequences; DNA base damage location and distribution; Ligation-mediated PCR

1 Introduction

Trinucleotide repeat (TNR) instability associated with human neurodegenerative diseases and cancer [1, 2] can be modulated through DNA metabolism and gene transcription [3–6]. Recent studies from our group and others have shown that DNA base damage and the base

excision repair (BER) pathway are actively involved in modulating somatic TNR instability [7–15] and promoting age-dependent somatic TNR expansion [16]. In our studies, we have designed synthesized oligonucleotide- and plasmid-based systems to study how DNA base lesions induce TNR instability through BER [17, 18]. Also, we have recently established an assay to detect DNA base lesions that occur in a long TNR tract in a site-specific manner with ligation-mediated polymerase chain reaction (LM-PCR) [19]. The methods allow for the determination of the position effects of DNA base lesions on TNR instability upon the specific damage locations [18] and distributions in TNR tracts.

For the oligonucleotide-based method, we have designed the oligonucleotide substrates that contained 20 TNRs with a base lesion (8-oxoguanine or abasic site, tetrahydrofuran, THF) located at 5'-side, middle or 3'-side of a TNR repeat tract (Figs. 1 and 2) [18]. These substrates are constructed by annealing a synthesized 100 nt-oligonucleotide containing 20 TNRs and a base lesion located at different locations of repeat tracts with its template strand. The template strand has a biotin residue attached at its 5'-end [20]. The principal of the method is to reconstitute BER by incubating TNR oligonucleotide substrates with purified BER core enzymes along with enzymes and proteins from other repair pathways to characterize the roles of BER and its interplay with other repair pathways in regulating TNR instability. Reconstituted BER removes a base lesion and results in the production of repaired products with different lengths of TNRs. The repaired products are then incubated with avidin–agarose beads allowing avidin to bind to a biotin residue attached to the 5'-end of the template strand. The repaired strand is separated from the template strand by denaturation of double-strand DNA with a low concentration of NaOH (0.15 M). The repaired products are recovered and amplified by PCR with a forward primer containing a fluorescence tag, 6-carboxyfluorescein (6-FAM) along with a reverse primer. The PCR-amplified fluorescence-labeled repair products are then subject to capillary electrophoresis and DNA fragment analysis for determination of the length change of TNRs in the repaired products (Fig. 1). The method is illustrated in a scheme shown in Fig. 1. Employing the approach, we have demonstrated that a base lesion located at the 5'-end of a TNR tract leads to repeat expansion, whereas a lesion located in the middle of the repeat tract causes deletion [18] (Fig. 2).

To determine how the distribution of multiple DNA base damage and single-strand DNA (ssDNA) breaks may govern TNR expansion and deletion, we have developed a plasmid-based in vitro method. This method employs a plasmid containing a (CAG)₃₅/(CTG)₃₅ tract, which is exposed to reactive oxygen species (ROS), specifically hydroxyl radicals produced by Fenton reaction (Fig. 3a). Hydroxyl radicals can induce ssDNA breaks and 8-oxoGs at multiple sites in CAG repeats that are repaired by BER. The site-specific distribution of ssDNA breaks in CAG repeats resulting directly from ROS and/or removal of hydroxyl radical-induced 8-oxoGs by BER, can be determined by LM-PCR (Fig. 3b). BER of base lesions in CAG repeats is reconstituted by incubating damaged plasmids with purified BER enzymes and cofactors. Repaired plasmids are then subject to PCR amplification with a 6-FAM-tagged forward primer and a reverse primer. Repaired products with different lengths of CAG repeats are separated by capillary electrophoresis, and repeat length is determined by DNA fragment analysis with the GeneMapper v. 5.0 software. The site-specific distribution of DNA base lesions in CAG repeats is then correlated with repeat expansion

and deletion. With this method, we have identified the hotspots and distribution of ssDNA breaks in a (CAG)₃₅ repeat tract that induced by hydroxyl radicals. We have found that hydroxyl radicals-induced ssDNA breaks predominantly distributed in the middle of the repeat tract, thereby leading to CAG repeat deletion via BER (Fig. 4).

2 Materials

2.1 TNR Oligonucleotide Substrates

Polyacrylamide gel purified synthesized oligonucleotides containing a TNR repeat tract with a base lesion at various locations in repeat tract.

1. Polyacrylamide gel purified synthesized oligonucleotide template strands that are tagged with a biotin residue at the 5'-end [20].
2. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, store at 25 °C.
3. 10 × DNA annealing buffer: 100 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, pH 7.5, store at -20 °C.

2.2 A Plasmid-Based TNR Substrate Containing a Long CAG Repeat Tract

1. 1 mM Fe²⁺ solution, freshly made and stored at 25 °C (*see Note 1*).
2. 1 mM EDTA, pH 8.0, freshly made and stored at 25 °C (*see Note 1*).
3. 100 mM sodium ascorbate, freshly made and stored at 25 °C (*see Note 1*).
4. 30% H₂O₂ stock solution, stored at 4 °C.
5. Thiourea-EDTA quenching buffer: 95 mM thiourea, 48 mM EDTA, pH 8.0, freshly made and stored at 25 °C.
6. pcDNA3.1/CT-GFP-TOPO-CAG₃₅ Plasmid: 1 µg/µL, stored at -20 °C.
7. 100% and 70% ethanol, stored at -20 °C.
8. 3 M sodium acetate, pH 5.2, stored at 25 °C (*see Note 2*).
9. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, stored at 25 °C.
10. 10 × TBE buffer: 1 M Tris base, 1 M boric acid, and 20 mM EDTA, stored at 25 °C.
11. 7.5 µg/µL linear acrylamide solution, stored at 25 °C.

2.3 Determination of ssDNA Breaks in CAG Repeats Using LM-PCR

1. Synthesized double-strand oligonucleotide DNA linkers (10 µM) (*see Fig. 3b*) and PCR primers (10 µM), stored at -20 °C.
2. 10 mM dNTP stock solution, stored at -20 °C.
3. 10 U/µL T4 polynucleotide kinase, stored at -20 °C.
4. 2 U/µL Deep Vent DNA polymerase, stored at -20 °C.

5. 10 × PCR enhancer solution, stored at −20 °C.
6. 400 U/μL T4 DNA ligase, stored at −20 °C.
7. 100 mM ATP stock solution, stored at −20 °C.
8. AmpliTaq Gold 360 DNA polymerase, stored at −20 °C.
9. 10 × AmpliTaq Gold 360 buffer, stored at −20 °C.
10. 3000 Ci/mmol ATP [γ -³²P] (10 mCi/mL), 100 μCi, stored at −20 °C.
11. 18% 8 M urea-denaturing polyacrylamide gel.
12. 2 × stopping buffer: 95% deionized formamide, 0.5 mM EDTA, pH 8.0, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF.

2.4 Reconstitution of BER

1. Purified recombinant human BER enzymes including DNA polymerase β (Pol β), AP endonuclease 1 (APE1), flap endonuclease 1 (FEN1), and DNA ligase I (LIG I), stored at −80 °C.
2. BER reaction buffer: 50 mM Tris-HCl, 50 mM KCl, 0.1 mg/mL BSA, 1 mM DTT, 0.1 mM EDTA store at 4 °C.
3. 10 mM dNTP stock solution, stored at −20 °C.
4. 50 mM Mg²⁺ stock solution, stored at −20 °C.
5. 10 × BER reaction stopping buffer: 100 mM EDTA, pH 8.0, stored at −20 °C.

2.5 Isolation of Repaired Products

1. Pierce Avidin-Agarose, 5 mL of settled gel (10 mL total slurry volume).
2. Binding Buffer: 0.1 M phosphate, 0.15 M NaCl, 1% Nonidet P-40, pH 7.2.
3. 3 M sodium acetate, pH 5.2, stored at 25 °C.
4. 100% and 70% ethanol, stored at −20 °C.
5. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, stored at 25 °C.
6. 7.5 μg/μL linear acrylamide solution.

2.6 Determination of TNR Instability

1. A polyacrylamide gel purified synthesized oligonucleotide control that contains a TNR tract without a base lesion (*see* Note 3).
2. Purified plasmid control without hydroxyl radical treatment (*see* Note 4).
3. Forward PCR primers tagged by a 6-FAM group: 10 μM, stored at −20 °C.
4. Reverse PCR primers: 10 μM, stored at −20 °C.
5. AmpliTaq Gold 360 DNA polymerase, stored at −20 °C.

6. 10 × AmpliTaq Gold 360 buffer, stored at −20 °C.
7. DNA size markers (MapMarker X-Rhodamine Labeled 50–1000 bp) for DNA fragment size determination through capillary electrophoresis.

3 Methods

3.1 Construction of TNR Oligonucleotide Substrates Containing a Synthesized DNA Base Lesion

1. Mix 50 pmol purified synthesized DNA oligonucleotide containing a TNR tract with a base lesion (8-oxoG or abasic site; a synthesized tetrahydrofuran residue can be used as an analogy of an abasic site), 100 pmol template strand, and 5 µL 10× annealing buffer in a 1.5 mL Eppendorf tube and add deionized water to a total volume of 50 µL.
2. Heat the annealing mixture in a heat block at 95 °C for 10 min and turn off the heat block until the temperature drops to room temperature (25 °C).
3. Dilute the substrate stock solution (1 µM) with BER reaction buffer to various concentrations for assembly of BER reactions according to the need of experiments.

3.2 Construction of a Plasmid-Based TNR Substrate Containing ssDNA Breaks at Multiple Sites in a Long CAG Repeat Tract

1. Make 30 µL DNA plasmid solution with 10 µg pcDNA3.1/CT-GFP-TOPO-CAG₃₅ plasmid in Tris buffer containing 50 mM Tris–HCl, pH 7.5.
2. Mix 10 µL of thiourea (0.4 M) with 32 µL EDTA (200 mM), pH 8 to make 42 µL quench solution (95 mM thiourea and 152 mM EDTA, pH 8).
3. Make 10 µL Fenton reaction solution: 0.01 mM Fe²⁺ and 0.02 mM EDTA, pH 8.0 (*see Note 1*).
4. Make 10 µL H₂O₂ solution (0.03%, 0.15%, 0.3%, 0.6%, and 0.9% H₂O₂) (*see Note 1*).
5. Mix DNA plasmid solution with 10 µL Fenton reaction solution, 10 µL 1 mM sodium ascorbate solution. Quickly add 10 µL H₂O₂ solution with various concentrations into each of reaction mixtures and incubate them in the dark at room temperature (25 °C) for 5 min.
6. Add 30 µL quench solution to the reaction mixture (total volume: 60 µL) and incubate at 25 °C for 30 min.
7. Add 6 µL 3 M sodium acetate (pH 5.2) and cold 120 µL 100% ethanol to the reaction mixture and mix well by gently tapping the bottom of the Eppendorf tube.
8. Freeze the reaction mixture at −80 °C for 30 min.

9. Pellet plasmid DNA by centrifugation at $11,269 \times g$ (Eppendorf Centrifuge 5417R) for 30 min.
10. Wash DNA plasmid pellets once with cold 70% ethanol.
11. Dry DNA plasmid pellets in the air for 5 min.
12. Dissolve DNA plasmid pellets in sterile water.
13. Measure the concentrations of DNA plasmids with Nano-Drop and store the plasmid DNA at -20°C for further analysis.
14. Check DNA strand breaks in the plasmids by running the plasmid samples (1 μg) in 1% agarose gel in $1\times$ TBE buffer through gel electrophoresis at 150 V for 1 h.

3.3 Identification of Site-Specific Distribution of ssDNA Breaks in a Long CAG Repeat Tract in a Plasmid Using LM-PCR

1. Phosphorylate the 5'-end of ssDNA breaks in a TNR tract in DNA plasmids (2 μg) by mixing 1 μL T4 PNK, with 2.5 μL $10\times$ PNK buffer, 2.5 μL 10 mM ATP. Add deionized water to a total volume of 25 μL . Incubate the reaction at 37°C for 1 h. Terminate the phosphorylation reaction and denature plasmid DNA at 75°C for 10 min.
2. Perform primer extension by incubating phosphorylated plasmids (25 μL) with 5 μL $10\times$ Thermopol reaction buffer, 1 μL of Deep Vent DNA polymerase (2 U), 0.8 μL of 10 mM dNTP, 4 μL of $10\times$ PCR Enhancer, 5 μL of 10 μM primer 1, 2 μL of 100 mM MgSO_4 . Add deionized water to 50 μL .
3. Perform the primer extension reaction in a PCR machine with the following program.
 - a. 95°C for 5 min.
 - b. 62°C for 30 min.
 - c. 72°C for 10 min.
4. Mix 9 μL (360 ng plasmid DNA) primer extension products with 1 μL of 10 μM double-strand linker DNA (see Fig. 3b), 2 μL of $10\times$ ligation buffer, 1.5 μL T4 DNA ligase (400 U/ μL), and 6.5 μL water and incubate the ligation mixture at 16°C overnight.
5. Mix 100 ng of the ligated DNA with 2.5 μL of 50 mM MgCl_2 , 2.5 μL of $10\times$ AmpliTaq Gold 360 buffer, 0.5 μL of AmpliTaq Gold 360 DNA polymerase (2.5 U), 0.8 μL of 10 mM dNTPs, 2.5 μL of 10 PCR Enhancer, 2.5 μL of 10 μM of Linker primer, 2.5 μL of 10 μM of primer 2. Add deionized water to make a total volume of 25 μL .
6. Perform PCR amplification with following the program for 40 cycles with a final extension at 72°C for 10 min.
 - a. 95°C for 5 min.

3.4 Reconstitution of BER

1. Mix purified BER enzymes, APE1 (10 nM), Pol β (10 nM), FEN1 (10 nM), LIG I (25 nM) with 50 nM synthesized oligonucleotide or plasmid substrates in BER reaction buffer with a total volume of 50 μ L.
2. Incubate reaction mixtures at 37 °C for 30 min (*see* Note 6).
3. Terminate BER reaction with the addition of 5 μ L 10 $\times$ BER stopping buffer.

3.5 Isolation of Repaired Products

1. To isolate repaired products resulting from BER with oligonucleotide substrates, mix 50 μ L reaction mixture with 30 μ L avidin–agarose beads and incubate the mixture at 25 °C for 2 h on a rotator.
2. Spin down avidin–agarose beads at $1957 \times g$ for 1 min, and remove the supernatant.
3. Wash the repaired products that are bound by avidin–agarose beads for three times with 50 μ L binding buffer.
4. Separate the repaired strands from their template strands by denaturation through incubation with 50 μ L 0.15 M NaOH for 15 min with rotation at 25 °C.
5. Spin down avidin–agarose beads that bind to the template strands at $1957 \times g$ for 2 min.
6. Carefully transfer 50 μ L supernatant containing the repaired strands to a new Eppendorf tube.
7. To isolate repaired products resulting from BER with plasmid substrates, skip **steps 1–6** and directly go to **step 8**.
8. Mix 50 μ L repaired products from oligonucleotide or plasmid substrates with 5 μ L 3 M sodium acetate (pH 5.2) and 100 μ L cold 100% ethanol.
9. Freeze the DNA precipitation mixture at -80 °C for 30 min.
10. Pellet DNA repaired products by centrifugation at $11,269 \times g$ for 30 min.
11. Wash the DNA repaired product pellets once with cold 70% ethanol.
12. Air-dry DNA pellets for 5 min.
13. Dissolve DNA pellets into the sterilized water and store at -20 °C.

3.6 Determination of TNR Instability

1. Mix 50 ng repaired products with 2 μ L 10 $\times$ AmpliTaq Gold 360 buffer, 0.5 μ L of AmpliTaq Gold 360 DNA (2.5 U), 2 μ L 10 μ M 6-FAM-tagged forward primer and 2 μ L 10 μ M reverse primer, 2 μ L 50 mM Mg^{2+} , 2 μ L 1 mM dNTPs and add sterile water to total volume of 20 μ L.
2. Perform PCR reaction with the following program for 30 cycles.

- a. 95 °C for 5 min.
 - b. 62 °C for 30 min.
 - c. 72 °C for 10 min.
3. Collect PCR products and store at –20 °C.
 4. Mix 2 µL PCR products with 1 µL MapMarker 1000 size markers and 9.5 µL Hi-Di formamide.
 5. Run the products through capillary electrophoresis to separate different sizes of DNA fragments with various lengths of TNR tracts.
 6. Analyze and determine the sizes of TNRs of repaired products using software GeneMapper v.5.0 (Life Technologies-ThermoFisher Scientific).

4 Notes

1. Solutions of Fenton reaction, sodium ascorbate, and H₂O₂ must be prepared freshly at the time when experiments are set up.
2. 3 M sodium acetate solution must be checked monthly to see if there is any crystal formed at the bottom of a solution bottle or tube. If any crystal is identified in the solution, fresh sodium acetate solution needs to be made.
3. Oligonucleotide control with TNRs must be carefully purified by 8 M urea-denaturing polyacrylamide gel electrophoresis through UV shadowing. It is important to use a relatively high percentage (18–20%) gel and avoid loading too much DNA in one lane of the gel.
4. It should be noted that purified pcDNA3.1/CT-GFP-TOPOCAG₃₅ plasmids contain both (CAG)₃₅ and (CAG)₃₄ repeats. This suggests that (CAG)₃₅ repeats were deleted during replication of the plasmids in *E. Coli* bacteria.
5. The watts and running time of polyacrylamide gel electrophoresis for separating multiple sizes of DNA fragments should be optimized based on the size of DNA products. Gel exposing time should be optimized based on the intensity of radioactivity.
6. The concentrations of BER enzymes can be varied to test the role of each individual repair enzyme in modulating TNR instability during BER.

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Fig. 1.

A scheme of the steps for determining the position effect of a DNA base lesion on TNR instability through BER with synthesized oligonucleotide substrates. A synthesized DNA base lesion is inserted at the 5'-end or in the middle of a TNR tract that contains 20 repeat units. The repeats are flanked by 20 nt random sequences at both the 5'- and 3'-side. Substrates are incubated with purified BER enzymes in the absence or presence of BER cofactors at 37 °C for 30 min allowing for BER of base lesions in the repeats that subsequently result in repeat instability. Unrepaired substrates and repair products with a 5'-biotin at the template strand are then incubated with avidin-agarose beads. The avidin beads are spun down and washed, and double-strand DNA is denatured by 0.15 M NaOH. The avidin-agarose beads attached to the template strand are then spun down, and the template strand is removed. The repaired strands with different lengths of TNRs, which stay in the supernatant are precipitated with ethanol and dissolved into water. Repair products are then

subject to PCR amplification with a 6-FAM-tagged forward primer and a reverse primer. The 6-FAM-labeled PCR products with various lengths of TNRs are separated by capillary electrophoresis. The lengths of TNRs are determined by DNA fragment analysis with the GeneMapper v5.0 software

Fig. 2. Experimental data showing the effects of a DNA base lesion on TNR instability during BER when it is located at the 5'-end (**a**) or in the middle of a (CTG)₂₀ tract (**b**) (reprinted from ref. [18] with a permission from PLoS One)

Fig. 3.

A schematic representation of the steps for determining the effects of the distribution of DNA base lesions in a long CAG repeat tract on repeat instability through BER. Plasmids containing (CAG)₃₅ repeats are incubated with Fenton reagents allowing for generation of hydroxyl radicals that subsequently create ssDNA breaks in the (CAG)₃₅ repeat tract. A portion of damaged plasmids is subject to LM-PCR, and PCR products are separated by denaturing urea polyacrylamide gel electrophoresis. The distribution of ssDNA in CAG repeats is determined by the mobility of PCR products according to the mobility of the DNA size markers. Another portion of damaged plasmids is subject to reconstituted BER allowing for production of BER repair products. Repair products are then amplified by PCR with a 6-FAM-tagged forward primer and a reverse primer. PCR products are separated by capillary electrophoresis. The sizes of CAG repeats in the repair products are determined by the GeneMapper v5.0 software

Fig. 4. DNA strand breaks results in CAG repeat instability through BER in a long repeat tract **(a)** DNA strand breaks in pcDNA3.1/CT-GFP-TOPO-CAG₃₅ plasmids induced by hydroxyl radicals generated by Fenton reaction at different percentage of H₂O₂ was detected by agarose gel electrophoresis. Lane 1 represents the DNA size markers. Lane 2 represents undamaged pcDNA3.1/CT-GFP-TOPO-CAG₃₅ plasmids. Lanes 3–7 indicate damaged pcDNA3.1/CT-GFP-TOPO-CAG₃₅ plasmids under treatment of Fenton reagents with various percentage of H₂O₂ (0.03–0.9%). **(b)** The results of urea-denaturing polyacrylamide gel electrophoresis of the products from LM-PCR, illustrate the multiple ssDNA breaks located at specific bases in 30 CAG repeats. The damaged bases are indicated by a series of white filled circles. The results showed that a low percentage of H₂O₂ at 0.03% resulted in the

localized distribution of ssDNA breaks in CAG repeats (lane 1). Increasing percentage of H₂O₂ led to increasing number of DNA damage sites and even distribution of ssDNA breaks in the CAG repeat tract. Lanes 1–5 indicate the LM-PCR results from damaged plasmids treated with Fenton reagents along with 0.03–0.9% H₂O₂. Lanes 6 and 7 are the markers for indicating the locations of specific DNA bases. (c) The instability of CAG repeats induced by hydroxyl radicals generated by Fenton reaction through BER. The results showed that little CAG repeat deletion and expansion products were detected from the untreated control plasmids. BER of ssDNA breaks in CAG repeats generated by Fenton reaction with 0.03% and 0.9% H₂O₂ led to a significant increase in the amount of repeat deletion products with (CAG)_{10–18} repeats