Mutation Research 775 (2015) 48–58

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

# A novel method for monitoring functional lesion-specific recruitment of repair proteins in live cells

Jordan Woodrick, Suhani Gupta, Pooja Khatkar, Kalpana Dave, Darya Levashova, Sujata Choudhury, Hadi Elias, Tapas Saha, Susette Mueller, Rabindra Roy*

*Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical School, Washington, DC 20057, United States*

ARTICLE INFO

*Article history:*
Received 16 December 2014
Received in revised form 17 March 2015
Accepted 27 March 2015
Available online 4 April 2015

*Keywords:*
DNA–protein interaction
Base excision repair
Abasic sites
Repair intermediate
Co-localization

ABSTRACT

DNA–protein relationships have been studied by numerous methods, but a particular gap in methodology lies in the study of DNA adduct-specific interactions with proteins *in vivo*, which particularly affects the field of DNA repair. Using the repair of a well-characterized and ubiquitous adduct, the abasic (AP) site, as a model, we have developed a comprehensive method of monitoring DNA lesion-specific recruitment of proteins *in vivo* over time. We utilized a surrogate system in which a Cy3-labeled plasmid containing a single AP-site was transfected into cells, and the interaction of the labeled DNA with BER enzymes, including APE1, Polβ, LIG1, and FEN1, was monitored by immunofluorescent staining of the enzymes by Alexafluor-488-conjugated secondary antibody. The recruitment of enzymes was characterized by quantification of Cy3-Alexafluor-488 co-localization. To validate the microscopy-based method, repair of the transfected AP-site DNA was also quantified at various time points post-transfection using a real time PCR-based method. Notably, the recruitment time kinetics for each enzyme were consistent with AP-site repair time kinetics. This microscopy-based methodology is reliable in detecting the recruitment of proteins to specific DNA substrates and can be extended to study other *in vivo* DNA–protein relationships in any DNA sequence and in the context of any DNA structure in transfectable proliferating or quiescent cells. The method may be applied to a variety of disciplines of nucleic acid transaction pathways, including repair, replication, transcription, and recombination.



## 1. Introduction

The study of DNA–protein relationships holds great biological significance and reveals previously unknown cellular phenomena. Understanding the intricacies of the interaction between important functional cellular proteins and the "blueprint" of the cell elucidates the mechanisms behind the regulation of cellular processes that may be important in understanding various diseases. DNA–protein interactions have been studied by numerous methods, but a particular gap in methodology lies in the study of DNA structure-specific interactions with proteins *in vivo*. Certain methods, including chromatin immunoprecipitation (ChIP) and ligation-mediated PCR (LM-PCR) are powerful methods to detect DNA–protein interactions *in vivo*. ChIP is useful for detecting interactions that are mediated by the sequence of the DNA while LM-PCR can determine the location and accumulation of certain DNA modifications in the DNA sequence, but information about the proteins interacting with those DNA modifications is, at best, indirect. Both methods are unhelpful for directly monitoring protein interactions that are mediated by a modification of the DNA structure, such as DNA adducts, DNA replication structures, or recombination structures [1–3]. Notably, much of the interactions of interest in the DNA repair field are mediated by modified, adducted DNA. Currently, several methods are available to observe DNA structure-specific interaction with associated proteins *in vitro*, including DNA footprinting, electophoretic mobility shift assay (EMSA), fluorescence anisotropy, and surface plasmon resonance (SPR), to name a few [4–8]. These techniques are valuable for dissecting mechanisms and quantifying kinetic parameters of the enzymatic interaction between a protein and a modified DNA structure, such as a DNA adduct. However, all the aforementioned methods fall short in elucidating the interaction of interest in live human cells, which is necessary for a complete physiological understanding that can be

*Abbreviations:* AP-site, abasic site; BER, base excision repair; APE1, apurinic apyrimidinic endonuclease 1; Polβ, DNA polymerase β; LIG1, DNA ligase 1; FEN1, flap endonuclease 1.

* Corresponding author at: Lombardi Comprehensive Cancer Center, Preclinical Science Building, LF09A, 3900 Reservoir Road, NW, Georgetown University Medical Center, Washington, DC 20057, United States. Tel.: +1 202 687 7390; fax: +1 202 687 1068.
*E-mail address:* rr228@georgetown.edu (R. Roy).

http://dx.doi.org/10.1016/j.mrfmmm.2015.03.013
0027-5107/

translated into useful knowledge about basic mechanisms and even human disease. The goal of the method developed and described in the present study is to monitor functional and specific recruitment of DNA repair proteins to adduct-containing DNA *in vivo*. In order to accomplish this goal, we analyzed repair of the abasic (AP) site, as a model.

AP sites are one of the most frequent non-coding lesions generated in cellular DNA, either by spontaneous or enzyme-catalyzed processes [9,10]. An abasic site is formed when the *N*-glycosyl bond between the base and deoxyribose sugar is hydrolyzed. This hydrolysis can occur during spontaneous depurination or as an intermediate step in the base excision repair (BER) pathway, which repairs small adducts in the cellular DNA. An AP-site is generated during BER after the initiating glycosylase excises a damaged base from the DNA structure. The resulting abasic site is subsequently cleaved at the first 5′ phosphate by apurinic/apyrimidinic endonuclease 1 (APE1), and repair is completed by gap-filling and ligation *via* other BER enzymes to fully restore the DNA sequence. The interaction of APE1 with abasic site DNA is well-characterized *in vitro*, both qualitatively and quantitatively [6,11–13]. However, as previously noted for general DNA–protein interactions, *in vivo* interactions between APE1 and AP-site DNA remain to be elucidated. At best, a quantification of abasic sites in the genomic DNA can be performed using aldehyde reactive probe (ARP), but one limitation is that ARP also detects incised AP-sites [10]. In fact, in a study where cleaved and intact abasic sites were quantified using a chemical method in human and rat tissues, almost all of detected AP-sites were incised, indicating that the interaction between APE1 and abasic sites occurs rapidly and that the current methods for detecting AP-sites are unable to capture the interaction [14].

Using co-localization, a fluorescence-based technique commonly used for monitoring protein–protein relationships, we developed a method for monitoring adduct-specific DNA–protein interaction *in vivo* using the interaction of APE1 with AP-site DNA as a model. We further validated this method by monitoring the recruitment of downstream BER proteins, including Polβ, LIG1, and FEN1, in a lesion-specific manner. Finally, we determined the functionality of the observed BER enzyme-lesion DNA co-localization by modifying some *in vivo* DNA repair assays developed previously by our lab [15,16].

## 2. Materials and methods

### 2.1. Construction of AP-site plasmid DNA

The preparation of the control and AP-site phagemid DNA was performed *in vitro* as previously described for 1,$N^6$-ethenoadenine-containing phagemids with some modification [15]. We engineered M13mp18 phagemids (7.2 kb) with a single tetrahydrofuran (THF) at the EcoRI restriction site in the multiple cloning site (MCS). Tetrahydrofuran is a stable analog of abasic site and is used in this study as a model AP-site. Typically, for each preparation of M13mp18-ctrl (no THF) or –AP (with THF) phagemid, 6 individual reactions were performed simultaneously according to the following protocol. Phosphorylation of the primers was performed by incubating 2 μg of THF-containing (5′-CCGAGCTCGXATTCGTAATC-3′) or control (5′-CCGAGCTCGAATTCGTAATC-3′) oligonucleotide (2 μl) with 1X PNK buffer, 400 nM ATP, 50 mM DTT, and 10 U of T4 polynucleotide kinase (New England Biolabs) in a 30 μL reaction volume at 37 °C for 45 mins. The phosphorylated oligonucleotide was purified through a G-25 column (GE Healthcare Life Sciences) according to the manufacturer's protocol. Then 6 μl of this purified oligonucleotide was incubated with 2 μg of M13mp18 ssDNA in an annealing buffer containing 10 mM Tris–HCl, pH 7.5, and 50 mM NaCl in a 20 μL reaction volume. This annealing reaction was incubated at 80 °C for 5 min and slowly cooled to room temperature with brief centrifugation when the reaction reached 50 °C. Then the annealing reaction was incubated with an extension reaction mixture containing 12 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$, 0.9 mM ATP, 0.9 mM of each dNTP, 6 mM DTT, and 100 μg/mL BSA, 10 U of T7 DNA polymerase (New England Biolabs), and 400 U of T4 DNA ligase (New England Biolabs) in a final reaction volume of 50 μL for 5 mins on ice followed by 5 mins at room temperature. The extension reaction was subsequently incubated at 37 °C for 1 h. After an hour, 50 nanomoles of ATP and 200 U of T4 DNA ligase were added to the extension reaction and incubated at 14 °C overnight for efficient ligation to occur. The 6 individual reactions were then pooled and incubated with 1X Supercoil-It buffer (Bayou Biolabs) and 2 μL of Supercoil-It enzyme mixture at 37 °C for 3 h. Phagemid DNA was recovered after the incubation by purification using Qiaquick PCR Purification kit (Qiagen). The DNA was eluted from the column using 50 μl of molecular grade water (DNase-, RNase-, and protease-free). Concentration of the eluted DNA was measured using a Nanodrop spectrophotometer, and the DNA was stored at −20 °C. Eighty five femtomoles (fmol) of each of the DNA constructs were treated with 20 nM APE1 (expressed and purified previously [17]) or 0.05 U of *Eco*RI and 10 U of *Sac*I. Digestion products were evaluated by gel electrophoresis on a 1% agarose gel. For experiments in Fig. 2D, an oligonucleotide containing Cy3 in the *Eco*R1 site was used for phagemid preparation (5′-CCGAGCTCGXATTCGTAATC-3′), where X = Cy3.

### 2.2. Construction of fluorescently labeled AP-site plasmid DNA

Similar to the previously described construction of AP-site phagemid DNA, fluorescently labeled AP-site or control phagemid DNA was prepared. During the polymerization step of the *in vitro* phagemid preparation, 1 nanomole of Cy3-dCTP (GE Life Sciences) was added to the dNTP pool generating a fraction of phagemids that were Cy3-labeled (control or AP-site-containing). In these constructs Cy3 was not placed at or around the assayed restriction site (*Eco*RI), whereas THF was placed in the *Eco*RI site. The quality of the constructs was evaluated as described in the above section. Quantification of Cy3 labeling of the phagemids was performed using an oligonucleotide containing a single Cy3 as a standard molecule to generate a standard curve and different dilutions of the phagemids. Quantification was carried out in a 96 well black plate with excitation at 480 nm and emission detected at 520 nm in a GloMax Multi-Detection system (Promega).

### 2.3. Transfection, harvest, and restriction digestion of AP-site plasmid DNA

Typically, 40 fmol of control or AP-site DNA was transfected into approximately 400,000 HCT116 cells in a well of a 6-well plate using 8 μL Lipofectamine 2000 (Life Technologies), according to manufacturer's protocol. At various time points post-transfection (5, 20, 30, 90, 150, and 300 min), the phagemid DNA was retrieved from the cells using the Qiagen Miniprep Kit (Qiagen) according to manufacturer's protocol. The retrieved phagemid DNA was aliquoted for 3 separate reactions: no digestion, *Sac*I digestion, and *Eco*RI digestion. Digestions were carried out according to New England Biolabs recommended protocols for the respective enzymes in 20 μL reaction volumes.

### 2.4. RNAi

Approximately 150,000 HCT116 cells were seeded on glass coverslips in the presence of gene-specific siRNA, Opti-mem low serum media (Life Technologies), and Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol for reverse