THE BULLETIN OF OKAYAMA UNIVERSITY OF SCIENCE No. 41 A pp. 51-57 (2005)

Nonisotopic assay for DNA repair apurinic/apyrimidinic endonuclease activity using fluorescein isothiocyanate-labeled oligonucleotide substrate and fluorescent image analyzer

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(Received September 2, 2005; accepted November 7, 2005)

Apurinic/apyrimidinic (AP) endonuclease plays a pivotal role in repair processing of abasic sites in damaged DNA via the base excision repair pathway. Quantitative assay of AP endonuclease in crude cell extract provides an important aspect for evaluation of the DNA repair potential of the cell. In the present study, we established a nonisotopic assay for measuring incision activity of AP endonuclease using fluorescein isothiocyanate-labeled oligonucleotide substrate and a fluorescent image analyzer. Using this procedure, the incision activity of endogenous AP endonuclease on a synthetic AP site (tetrahydrofuranyl moiety) of an oligonucleotide can be measured in crude extracts from *Escherichia coli* and *Schizosaccharomyces pombe* cells. The activity of exogenously expressed human enzyme (hAPE1) in AP endonuclease-deficient bacterial and yeast cells can also be detected.

1. Introduction

Cells tolerate continuous DNA base damage that disrupts their viability and genomic stability [1,2]. DNA base excision repair (BER) is a key pathway for restoring the chemical integrity of DNA and requires the action of at least four enzymes: a damage-specific DNA glycosylase, followed by the apurinic/apyrimidinic (AP) endonuclease, DNA polymerase and DNA ligase [3-5]. Detection of base lesion by the DNA glycosylase initiates BER and creates abasic sites in DNA. More than 10,000 AP sites per day are generated in each human cell, both spontaneously and by DNA glycosylases [2,6]. AP sites are unstable, spontaneously degrading into abnormal DNA strand breaks and this disrupts cellular processes. AP sites retard DNA polymerase, cause base misincorporation and are highly mutagenic at the transcription level [1,4,6]. In humans, the major AP endonuclease (APE1) cleaves the phosphodiether bond 5' of the AP sites, priming repair synthesis by DNA polymerase [3-5]. Thus, AP endonuclease plays a pivotal role in repair processing of deleterious AP sites in the BER pathway.

Quantitative measurement of AP endonuclease activity in cell extracts is important for evaluation of the DNA repair potential of the cell. Incision of the abasic site by AP endonuclease has been analyzed using AP-site containing oligonucleotide substrates and polyacrylamide gel electrophoresis under denaturing conditions. In these experiments, the oligonucleotides are generally labeled with radioactive ^{32}P at their 5'- or 3'-ends, and detected by autoradiography or radioisotopic imaging with a Phosphor Imager. However, development of a

nonisotopic technique will avoid the cumbersome procedures and short half-lives associated with radioactive materials. Recent fluorescent image analyzer models can provide the sensitive detection of fluorescein isothiocyanate (FITC)-labeled DNA in polyacrylamide gel at levels of 0.1 femtomole (fmol) per DNA band. In the present study, we established a non-radioactive assay for measuring AP endonuclease activity using FITC-labeled oligonucleotide and a fluorescent imager. Using this procedure, endogenous AP endonuclease activities can be measured in crude cell extracts of *Escherichia coli* and *Schizosaccharomyces pombe* cells. Exogenously expressed hAPE1 in bacterial and yeast cells can also be detected.

2. Materials and Methods

2.1. Materials

Oligonucleotide containing tetrahydrofuranyl (THF) lesion and FITC molecule at the 5'-end, and complementary strand were custom synthesized by Hokkaido System Science Co., Ltd. (Sapporo, Japan) and purified by HPLC (Fig. 1). THF phosphoramidite (dSpacer) was purchased from GLEN RESEARCH (Sterling, VA). The oligonucleotide strand was annealed to the complementary strand in 10 mM Tris-HCl, 1 mM EDTA and 33 mM NaCl (pH 8.0) by heating at 90°C for 2 min for denaturation and cooling to room temperature overnight. *E. coli* endonuclease IV (Endo IV) was purchased from R&D Systems, Inc. (Minneapolis, MN). One unit is defined as the amount of enzyme required to cleave an AP site oligonucleotide within an oligonucleotide duplex at a rate of 1 pmol/h at 37°C.

2.2. Cells

E. coli AB1157 (*thr-1 ara-14 leuB6* (Am) *lacY1 D* (*gpt-pro A2*) *62 tsx-33 supE44* (Am) *galK2 rec hisG4* (Oc) *rfbD1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3* (Oc) *thi-1*) and its derivative RPC501 Δ(*xthA-pncA*) *nfo-1*::*kan* [7] were used for preparation of cell extracts and expression of hAPE1. The wild-type *S. pombe* used in this study was ED0665 (ATCC no. 96993; *h*⁻, *ade6*-M210, *leu1*-32, *ura4*-D18). An *apn2*-null strain (*apn2*Δ) was generated as follows. The gene cassette for disruption of the *apn2* gene was produced by cloning the *ura4*⁺ fragment into a pGEM-T plasmid with the *apn2* genomic sequence. Briefly, the pUR18 plasmid was digested with *Cla* I and blunt-ended with KOD polymerase (TOYOBO, Osaka, Japan). A 1.7-kb fragment containing the *ura4*⁺ gene was excised by *Hind* III digestion and subcloned into pGEM-apn2 digested with *Bsa* BI and *Hind* III. The *apn2*::*ura4* DNA fragment was excised from the plasmid by *Xmn* I and *Sac* I digestion, separated from the vector by electrophoresis, and used to transform haploid strain ED0665. Finally, *ura*⁺ clones were selected by PCR and Southern analysis.

2.3. Construction of hAPE1 expression plasmid

pUC19 was used for expression of hAPE1 in *E.*

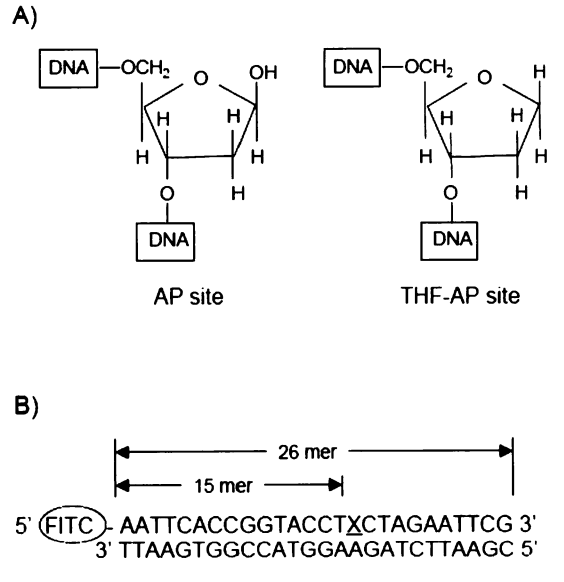

Fig. 1. Substrate for AP endonuclease assay. (A) Chemical structure of the natural AP site (left) and synthetic THF AP site (right). (B) Nucleotide sequence of the oligonucleotide duplex. X in the upper strand is the THF residue.

coli cells. The cDNA fragment of hAPE1 was amplified by PCR from a pUAEH1 plasmid [8] using a gene specific primer (5'-ccc aag ctt CCG AAG CGT GGG AAA AAG-3') and the universal primer M4 for pUC plasmid. The PCR product was digested with *Hind* III and *Sal* I and was inserted into a pUC19 plasmid previously digested with the same enzymes. The resultant recombinant plasmid was then introduced into *E. coli* RPC501. pAUR224 (Takara Bio Inc., Otsu, Shiga, Japan) is an *S. pombe* expression vector that includes the cytomegalovirus promoter and the aureobasidin A-resistant gene (*aur1'*) as a selection marker. hAPE1 cDNA was amplified by PCR from a pUAEH1 plasmid using a gene specific primer pair (5'-gac tcg aga tgC CGA AGC GTG GGA AAA AG-3' and 5'-tag gat ccC AGT GCT AGG TAT AGG G-3'). The PCR product was digested with *Xho* I and *Bam* IH and was inserted into a pAUR224 plasmid previously digested with the same enzymes. The resultant recombinant plasmid was then introduced into *S. pombe apn2*Δ cells.

2.4. Preparation of whole cell extract

E. coli strains were cultured in LB medium containing appropriate antibiotics at 20°C overnight. After bacteria were harvested by centrifugation, they were resuspended in 10 volumes of buffer A (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol and 0.02% Triton X-100), and sonicated (6 × 30 s) on ice at full power using an ultrasonic disruptor (UD-201; TOMY SEIKO Co., Ltd, Tokyo, Japan). After the sonicate was centrifuged (30 min at 100,000 × g), the supernatant was used for AP endonuclease assay. *S. pombe* strains were cultured in YE medium (0.5% yeast extract and 3% glucose) containing 0.3 μg/ml aureobasidin A (Takara Bio) at 28°C overnight. After yeast cells were harvested by centrifugation, they were resuspended in 5 volumes of buffer B (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M NaCl, 5% glycerol, 3 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), and were then disrupted using a French press (15000 p.s.i.). After the sonicate was centrifuged (30 min at 100,000 × g), the supernatant was used for AP endonuclease assay. Proteins were quantitated by dye-binding method (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

2.5. AP endonuclease assay

Purified enzyme or cell extract was incubated

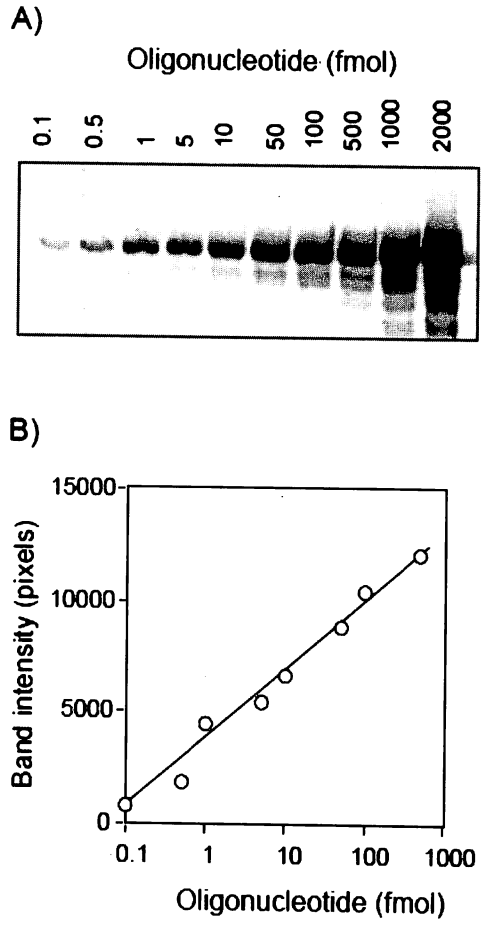

Fig. 2. Detection of FITC-labeled oligonucleotide by FMBIO. (A) Various amounts of oligonucleotide duplex diluted with TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 100 μg/ml of sonicated calf thymus DNA were electrophoresed in denatured polyacrylamide gel as described in section 2.5. Gel was scanned by FMBIO to visualize the FITC-labeled oligonucleotide bands. (B) Intensity of each band was plotted against the $\log_{10}$ of the amount of oligonucleotide.

with 50 nM (50 fmol/μl) FITC-oligonucleotide substrate in buffer (10 μl) containing 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, 50 mM NaCl and 1 mM dithiothreitol at 37°C for the times indicated in the Figures. After stopping the reaction by addition of 6 μl of formamide, the oligonucleotide duplex was denatured at 95°C for 5 min and was then loaded on a 20% denaturing polyacrylamide gel in 7 M urea and 1×TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA). The gel (W×H×D = 180 mm × 200 mm × 0.5 mm) was cast using electrophoresis apparatus (AE-6141; Atto Co., Tokyo, Japan). Electrophoresis was performed using 0.5×TBE electrode buffer at a constant 20 mA until the xylene cyanol dye marker migrated to about 120 mm. FITC-labeled oligonucleotides were detected using the FMBIO II Fluorescent Image Analyzer (Hitachi Software Engineering Co., Ltd., Yokohama, Japan). The FITC fluorophore is excited by a solid-state laser at 495 nm, and emits a fluorescent light signal at 521 nm, which is then isolated using a 505-nm filter (channel 2). Repeating time for the fluorescent reading was set at 800 times. After capturing, the intensity of each band was determined using NIH image (v. 1.62).

3. Results and Discussion

3.1. Nonisotopic substrate for AP endonuclease

FITC-labeled oligonulceotide was employed as a substrate for nonisotopic assay of AP endonuclease activity (Fig. 1). Oligonucleotides containing natural AP sites are generally prepared by treating a uracil-containing oligonucleotide with uracil DNA glycosylase. However, the resultant AP site is labile and the oligonucleotide is automatically cleaved at the site by a β-elimination reaction. This type of substrate must therefore be prepared just prior to use. On the other hand, oligonucleotides containing synthetic AP sites (THF moieties) are stable and good substrates for AP endonuclease, which can hydrolyze 5' of the AP site [9]. A reagent able to automatically insert THF moieties during DNA synthesis is commercially available. In this study, THF-oligonucleotides were labeled with FITC at the 5'-end, which is readily and sensitively detected by the FMBIO II Fluorescent Image Analyzer. Various amounts of oligonucleotide duplex were electrophoresed under denatured conditions, and the gel was scanned using the FMBIO analyzer to visualize the oligonucleotide bands (Fig. 2). The

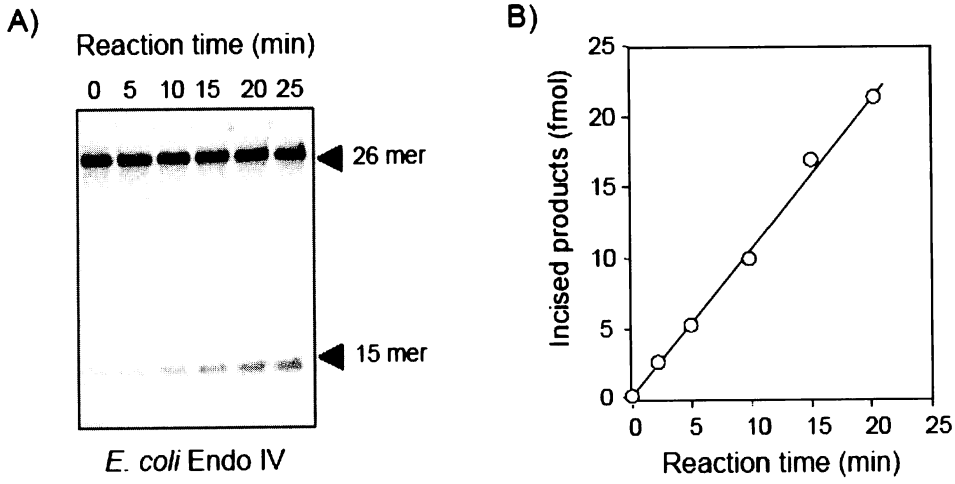

Fig. 3. AP endonuclease assay of *E. coli* Endo IV. (A) *E. coli* Endo IV (2×10^{-4} units) was allowed to react with THF-containing oligonucleotide at 37°C for the times indicated. (B) After quantification of the products, incision activity was expressed graphically.

smallest amount of FITC-labeled oligonucleotide (0.1 fmol = 10^{-16} mol) was detected as a single band. The intensity of each band increased linearly against the $\log_{10}$ of the oligonucleotide level.

3.2. AP endonuclease assay of *E. coli* Endo IV

Purified *E. coli* Endo IV was employed for establishing the AP endonuclease assay using FITC-labeled oligonucleotides (Fig. 3). The substrate concentration in the reaction mixture was fixed to be 50 nM based on the known *Km* value (16 nM) of hAPE1 for THF-containing substrate [9]. After the reaction, a 15-mer oligonucleotide was released from the 26-mer oligonucleotide substrate. This indicates that the Endo IV hydrolyzed 5' of THF moiety in the oligonucleotide substrate. The incised products increased linearly with reaction time. This indicates that the nonisotopic assay allows quantitative measurement of AP endonuclease activity.

3.3. AP endonuclease assay using whole cell extracts from bacterial and yeast cells

E. coli has two genes encoding AP endonuclease, *xth* and *nfo*, and the gene products are exonuclease III (Exo III) and Endo IV, respectively [7]. Whole cell extracts from the wild-type strain (AB1157) and an AP endonuclease-deficient strain (RPC501, *xthΔ/nfoΔ*) were prepared, and AP endonuclease activities in the extracts were measured using the nonisotopic assay system (Fig. 4). The incised products increased with the amount of extract from wild-type cells. On the other hand, no AP endonuclease activity was observed in the extract from *xthΔ/nfoΔ* cells, even when the amounts of *xthΔ/nfoΔ* extract in the reaction were 10-fold higher than those of the wild-type extract. This indicates that the incised products are responsible for Exo III and Endo IV activity and that the reaction system allows measurement of endogenous AP endonuclease activity in *E. coli*, even in whole cell extract. Human APE1 cDNA was exogenously expressed in

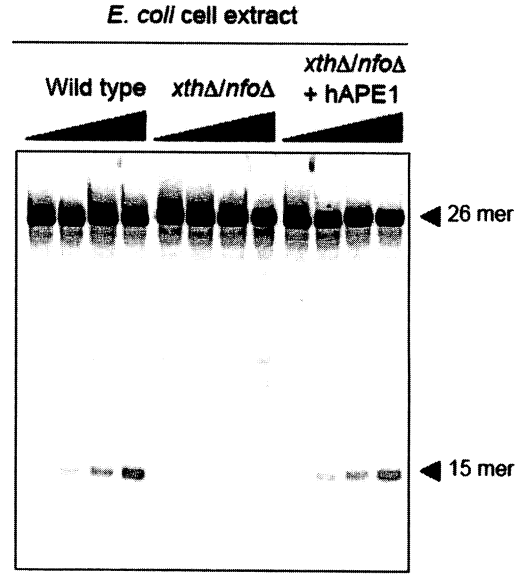

Fig. 4. AP endonuclease assay using whole cell extract of *E. coli*. Whole cell extracts were prepared from AB1157 cells (wild type), RPC501 cells (*xthΔ/nfoΔ*) cells and RPC501 expressing hAPE1 (*xthΔ/nfoΔ* + hAPE1) as described in section 2.4. The increasing amounts of whole cell extract were allowed to react with THF oligonucleotide at 37℃ for 10 min. The amounts of protein used for assay were as follows: Wild type, 0, 0.005 μg, 0.01 μg and 0.02 μg; *xthΔ/nfoΔ*, 0, 0.05 μg, 0.1 μg and 0.2 μg; and *xthΔ/nfoΔ* + hAPE1, 0, 0.05 μg, 0.1 μg and 0.2 μg. Note that the amounts of extract from wild type cells are 10-fold less than those from *xthΔ/nfoΔ* cells and *xthΔ/nfoΔ* + hAPE1 cells.

xthΔ/nfoΔ cells and incision of AP sites by hAPE1 was measured. The 15-mer incised products responsible for hAPE1 were detected with increasing amounts of extract.

Apn2p is the major AP endonuclease activity from *S. pombe* and is involved in the BER pathway [10-12]. The *apn2*-null mutant was generated by gene disruption, and the AP endonuclease activity of the mutant was compared with wild-type cells (Fig. 5). The 15-mer products generated by incision of the AP site were observed in with wild-type extract. In addition to the normal product, a 14-mer band was also observed, probably due to chewing by exonuclease(s) in crude yeast extract. No AP

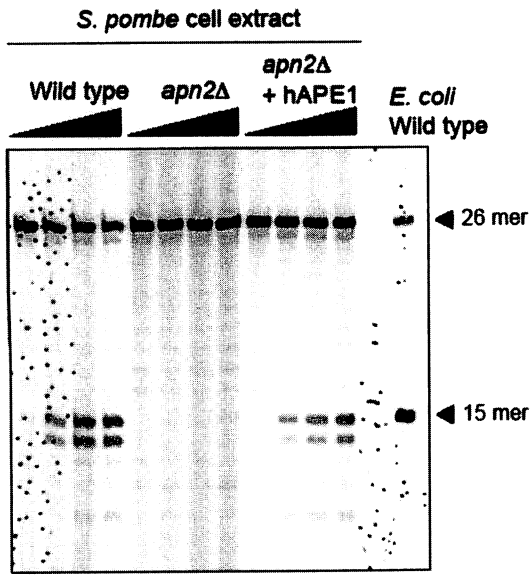

Fig. 5. AP endonuclease assay using whole cell extract from *S. pombe*. Whole cell extracts were prepared from ED0665 cells (wild type), *apn2*-null cells (*apn2*Δ) cells and *apn2*-null cells expressing hAPE1 (*apn2*Δ + hAPE1) as described in section 2.4. The whole cell extract (5 μg protein) from each strain was allowed to react with THF oligonucleotide at 37°C for 0, 10 min, 20 min and 30 min.

endonuclease activity was observed in the extract from *apn2*Δ cells. This result coincides with previous observations by Ribar *et al.* [11]. Exonuclease activity in crude yeast extract is likely higher than in bacterial extract. Human APE1 cDNA was expressed in *apn2*Δ cells and the AP endonuclease activity was measured. The 15-mer incised products responsible for the exogenously expressed hAPE1 were detected with increasing amounts of extract.

Our non-radioactive procedure allows detection of endogenous AP endonuclease activity in the crude extracts of bacterial and yeast cells, as well as exogenously expressed human enzyme. The non-radioactive assay provides an alternative to the ^{32}P-based system for simple detection of DNA repair activity including DNA glycosylases and AP endonuclease in crude cell extracts. Kreklau *et al.* [13] described a similar non-radioactive assay to measure various DNA glycosylases and AP endonuclease activities in extracts of cultured mammalian cells. They used hexachlorofluorescein-labeled oligonucleotides with various lesions, including O^6-methylguanine, ethenoadenine, 8-oxoguanine and THF.

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