**Highly Sensitive Apurinic/Apyrimidinic Site Assay Can Detect Spontaneous and Chemically Induced Depurination under Physiological Conditions**

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**Abstract**

One of the most prevalent lesions in DNA is the apurinic/apyrimidinic (AP) site, which is derived from the cleavage of the N-glycosyl bond by DNA glycosylase or by spontaneous depurination. AP sites are repaired by AP endonucleases during the process of base excision repair; however, an imbalance in this DNA repair system may cause mutations as well as cell death. We have established a sensitive and convenient slot-blot method to detect AP sites in genomic DNA using a novel aldehyde reactive probe (ARP), which reacts with the aldehydic group of ring-opened AP sites. The reaction of 1 μM of ARP with 15 μg of genomic DNA containing AP sites at 37°C was completed within 1 min. The AP site-ARP complex was remarkably stable during incubation in TE buffer, even at 100°C for 60 min. The sensitivity of this assay enables detection of 2.4 AP sites per 10^7 bases. By using this ARP-slot-blot assay, the rate of spontaneous depurination of calf thymus DNA was determined. Under physiological conditions, AP sites were increased at 1.54 AP sites/10^8 nucleotides/day (9000 bases). By using this ARP-slot-blot assay, the rate of spontaneous depurination of calf thymus DNA was determined. Under physiological conditions, AP sites were increased at 1.54 AP sites/10^8 nucleotides/day (9000 bases). This highly sensitive assay allows us to determine the endogenous level of AP sites in genomic DNA, as well as to investigate whether DNA-damaging agents cause imbalances of base excision/AP endonuclease repair in vivo and in vitro.

**Introduction**

Various DNA adducts are produced by electrophilic chemicals such as alkylating agents. In addition, highly sensitive methods have enabled detection of endogenous adducts in DNA extracted from tissues of experimental animals as well as humans (1). Many of these DNA lesions are repaired by a base excision repair pathway (2–6). During the first process of base excision repair, DNA glycosylase cleaves a modified DNA base at the glycosyl bond, resulting in formation of an AP site. AP sites can also be caused by spontaneous depurination of labile DNA adducts as well as unmodified bases.

Formation of AP sites is a relatively frequent event in chromosomal DNA under physiological conditions (7). AP sites inhibit DNA replication and also result in base substitution mutations and loss of genetic integrity (8). In a recent study, it was proposed that an imbalance in the base excision repair pathway causes mutational events and may play an important role in carcinogenesis (9).

Several methods are presently available to detect AP sites (10–16); however, these methods are either not direct measurements, insensitive to measure low numbers of AP sites, or difficult to conduct. Recently, a novel reagent for measuring AP sites was prepared by reacting O-(carboxymethyl)hydroxylamine with biotin hydrazide in the presence of carboximide (15). This reagent, called ARP, is a specific biotin-tagged probe that reacts with aldehydic ring-opened AP sites (15, 16). The number of biotin-tagged AP sites can then be determined colorimetrically by an ELISA-like assay. The present study reports a newly developed and highly sensitive method for the detection of AP sites in genomic DNA by a combination of ARP and slot-blot techniques.

**Materials and Methods**

Aldehyde Reactive Probe-Slot-Blot. The protocol of ASB uses the slot-blot method and ARP to measure AP sites (15). Fifteen μg of DNA in 150 μl of PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH_2PO_4·7H_2O, and 1.4 mM KH_2PO_4, pH 7.4) was incubated with 1 μM ARP (Dojindo Laboratories, Kumamoto, Japan) at 37°C for 10 min. The number of AP sites in the internal standard DNA was determined by the microplate method with ARP by Dr. Kubo (University of Osaka Prefecture, Sakai, Japan). After precipitation using cold ethanol, DNA was washed with 70% ethanol and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.2) at 3 μg/100 μl. The internal standard was serially diluted with calf thymus DNA (3 μg/100 μl TE buffer). DNA was heat-denatured at 100°C for 5 min, quickly chilled on ice, and mixed with an equal amount of 2 μM ammonium acetate. The single-stranded DNA was then immobilized on a BAS-85 NC membrane (Schleicher and Schuell) using a MiniFold II vacuum filter device (Schleicher and Schuell). The slots were rinsed with 200 μl of 1 mM ammonium acetate. The NC membrane was soaked with 5X SSC (0.75 M NaCl, 0.075 mM trisodium citrate) at 37°C for 15 min and then washed overnight in a vacuum oven at 80°C for 1–2 h. The membrane was washed with 1 M NaCl in 1 mM EDTA, 0.5% casein, 0.25% BSA, and 0.5% Tween 20 at room temperature for 30–45 min. After rinsing the NC membrane with washing buffer (0.26 M NaCl, 1 mM EDTA, 20 mM Tris-HCl, and 0.1% Tween 20, pH 7.5) for 15 min, the enzymatic activity on the membrane was visualized by the ECL reagents (Amersham Corp.). The NC filter was then exposed to X-ray film (Kodak XAR 5X, Kodak) for 5–15 s. The developed film was analyzed using a Ultrascan XL scanning densitometer (Pharmacia) and GelScan XL software (Pharmacia). Quantitation was based on comparisons to internal standard DNA containing the known amount of AP sites.

**AP Site Preparation by Heat/Acid Condition.** AP sites were produced in calf thymus DNA by heat-acid-buffer solution. Intact calf thymus DNA (Sigma Chemical Co.) or calf thymus DNA pretreated with 5 mM methoxyamine (Sigma) was added to sodium citrate buffer (10 mM sodium citrate containing 10 mM NaH_2PO_4 and 10 mM NaCl, pH 5.0) and held at 70°C for various lengths of time. The reaction was stopped by chilling rapidly on ice, and the DNA was then precipitated with cold ethanol, washed once with 70% ethanol, dried, and resuspended in sterilized distilled water.

**Treatment with hAPE and NaOH.** Heat-acid-treated calf thymus DNA (15 μg) and 23 ng of hAPE (a gift from Dr. M. Kelley, Indiana University, Indianapolis, Indiana) in 67.5 μl of 10 mM Tris-HCl/KOH buffer (pH 7.5) containing 50 mM NaCl and 5 mM MgCl_2, were incubated at 37°C for 30 min. For alkaline treatment, 1/10 volume of 2 M NaOH was added to the above reaction mixture and then incubated at 37°C for 15 min. The samples were precipitated with cold ethanol as described above, followed by resuspension with PBS for ASB assay.
AP Site Induction by Treatment with MMS. H2E1 cells, a human B-lymphoblastoid line immortalized with EBV expressing human Cyp 2E1, were generously provided by Gentest Corp. H2E1 cells were grown in RPMI 1640 supplemented with l-glutamine, HEPES buffer, NaHCO₃, l-histidinol, and 10% FCS (17). Cells (5.0 × 10⁵) per ml were exposed in triplicate to MMS (0.05—1.5 mM) at 37°C for 24 h. Following treatment, the cells were pelleted and washed once by spinning for 4 min at 1000 rpm. For determining toxicity of treatment, the cells were stained by trypan blue. The cell pellets were then frozen at −70°C before using.

DNA Extraction. DNA was extracted by a procedure modified from the method reported by Gupta (18). After thawing the frozen cells, the pellets were incubated in lysis buffer (Applied Biosystem) overnight at 4°C with proteinase K (500 µg/ml, Applied Biosystem). DNA was then extracted twice with a mixture of phenol/chloroform/water (Applied Biosystem) at 4°C and once with Sevag (chloroform:isoamyl alcohol, 24:1), followed by ethanol precipitation. The extracted DNA was resuspended in sterilized PBS (pH 7.4) and incubated at 37°C for 30 min with a mixture of RNase T1 (50 units/ml) and RNase A (100 µg/ml). After extraction with Sevag, DNA was precipitated with ethanol and then resuspended in sterilized distilled water. The DNA solution was stocked at −70°C for ASB assay.

Results

Sensitivity of the ASB Method. Heat/acid-treated calf thymus DNA containing 800 AP sites/10⁶ nucleotides, incubated with 1 mM ARP, was serially diluted with unmodified calf thymus DNA. A typical X-ray film from the ASB assay with 3 µg of DNA/slot is shown in Fig. 1A. The slot density decreased with dilution of ARP-complex. The relationship between density, as measured by a scanning densitometer, and the concentration of AP sites is shown Fig. 1B. The sensitivity of ASB assay was typically 0.24 AP sites/10⁶ nucleotides.

Optimum Conditions of ASB. The ability of ARP reagent to react with AP sites in calf thymus DNA was examined. The reactivity was measured by incubation with various concentrations of ARP ranging from 1 µM to 10 mM, as well as different reaction times. The ARP reaction at 37°C for 10 min was enhanced by increasing the concentration of ARP reagent, which reached saturation at 1 mM or more (Fig. 2A). The rate of reaction of 1 mM ARP to AP sites was extremely rapid, with ARP incorporation into DNA accomplished within 1 min at 37°C.

Before immobilizing DNA on the NC membrane, it was necessary to denature the DNA after ARP conjugation. To evaluate the influence of denaturation on the stability of the ARP complex, the persistence of the ARP complex at various incubation times at 100°C was measured using DNA samples containing 60, 120, and 800 AP sites/10⁶ nucleotides. The ARP complex was stable at 100°C for at least 60 min. We varied the amount of DNA loaded on the NC membrane to study the response of the ASB assay. A DNA sample containing 800 AP sites/10⁶ nucleotides was serially diluted with TE buffer, and different amounts of DNA were applied to the NC filter. We observed a linear relationship between slot density and the amount of DNA (Fig. 2B).

Effect of hAPE on ASB Assay. To examine whether AP sites were detectable with the ASB assay after incision by AP endonuclease, calf thymus DNA treated with heat/acid-solution was incubated with hAPE, which incises the DNA 5' to the AP site. After 30 min of incubation, hAPE reduced AP sites in the DNA approximately 28% (Fig. 3). In addition, the combination of hAPE and 0.2 M NaOH, which cleaves the DNA 5' and 3' to the AP site, decreased the number of AP sites 70%. In contrast, no reduction of AP sites was observed after treatment with 0.2 M NaOH, which incises DNA 3' to the AP site.

AP Site Induction at Heat/Acid and Physiological Conditions. AP sites in calf thymus DNA were reduced by treatment with 5 mM methoxyamine and then created by incubating in sodium citrate buffer (pH 5.0) at 70°C for up to 150 s. The number of AP sites increased in proportion to the length of incubation. Linear regression revealed that approximately 10 AP sites/10⁶ nucleotides were produced per min by incubation at 70°C at pH 5.0 (Fig. 4A). To determine the rate of spontaneous depurination of DNA under physiological conditions, methoxy-amine-pretreated calf thymus DNA was incubated at 37°C in PBS (pH 7.4) for up to 10 days. The increase in AP sites was proportional to the incubation time. These data showed that approximately 1.54 AP sites/10⁶ nucleotides were produced per day (Fig. 4B).

AP Site Induction by MMS. H2E1 cells were exposed to MMS at different concentrations ranging from 0.05 to 1.5 mM at 37°C for 24 h. Four AP sites/10⁶ nucleotides were detected in control H2E1 cells. AP sites in DNA increased two and five times compared with control at 0.5 and 1.5 mM, respectively (Fig. 5). In contrast, there was no increase in AP sites at 0.15 mM or less. In this experiment, cytotoxicity evaluated by trypan blue dye exclusion showed high survival rates (95% or more) after exposure to 0.05—0.5 mM MMS. Cell survival was more than 90% following exposure to 1.5 mM.
ARP-SLOT-BLOT ASSAY TO DETECT AP SITES

because there was no reaction between normal nucleosides and ARP during a 4-day incubation period at 37°C (16), the number of AP sites detected in the control H2E1 cells appears to be due to endogenous AP sites in genomic DNA. Therefore, these data indicate that the sensitivity of the

Discussion

Sensitivity of ASB Method. The present study reports the development of a highly sensitive, specific, and convenient method to detect AP sites in genomic DNA at a detection limit of 0.24 AP sites/10^6 nucleotides. The sensitivity of the ASB assay, which is based on the combination of a slot-blot method and ARP reagent, is one or two orders of magnitude higher than presently available methods of detecting AP sites (10–16). It has been estimated that the rate of AP site formation is 10,000 per cell per day in mammalian cells (7). In this previous report, the rate of depurination of double-stranded DNA was estimated using the depurination rate of DNA at 70°C and physical chemistry. This methodology was not sensitive enough to detect directly the slow rate of depurination at 37°C and pH 7.4. In the present study, the ASB assay clearly demonstrated spontaneous increases in AP sites, which are due to depurination, at 1.54 AP sites/10^6 nucleotides/day (9,000 AP sites/cell/day) at 37°C and pH 7.4. The number of AP sites present in genomic DNA under physiological conditions or AP site kinetics following exposure to low levels of DNA-damaging agents is not known, due in part to the instability of AP sites and insensitivity of AP site assays. In this report, we demonstrated four AP sites/10^6 nucleotides in control H2E1 cells (23,000 AP sites/cell).
ABSTRACT

The DNA backbone. The reason why hAPE treatment alone reduced the hAPE and/or NaOH at 37°C. There was no reduction in the number of cleavage relative to AP sites. To assessing AP endonuclease activity as well as determining the location of nicks 3' to AP sites during the incubation of DNA with heat/acid treatment. heat-labile DNA adducts as well as DNA glycosylase activity. The ASB assay including ARP reagent are presently commercially available. The ASB assay will be useful for measuring the amount of base excision repair pathway may play an important role in carcinogen induction with NaOH (19). In contrast, 5' incision by hAPE and both 5' and 3' nicks to AP sites by combination of hAPE and NaOH reduced the number of AP sites by 28 and 70%, respectively. The marked decrease in the number of AP sites appears to be due to the release of AP sites from the DNA back bone. The reason why hAPE treatment alone reduced the number of AP sites on the DNA may be due to the spontaneous induction of nicks 3' to AP sites during the incubation of DNA with heat/acid-solution. This experiment suggests that this assay may also be applicable to assessing AP endonuclease activity as well as determining the location of cleavage relative to AP sites.

ADVANTAGES OF ASB ASSAY. As described above, the ASB assay is a highly sensitive and specific method to detect AP sites. There are other advantages of the ASB over other methods for quantitation of AP sites. This method uses small amounts of genomic DNA (15 µg) isolated from any type of cell and tissues or enzyme-digested DNA fragments with length more than 300 nucleotides without any prelabelling process. No radioactive materials are required, and 28 samples can be analyzed in 1 or 2 days. Moreover, all materials required for the ASB assay including ARP reagent are presently commercially available. The ASB assay will be useful for measuring the amount of heat-labile DNA adducts as well as DNA glycosylase activity.

BIOLICAL SIGNIFICANCE OF AP SITES. The present study demonstrated that the ASB assay can detect even a slight increase in AP sites caused by heat/acid treatment or MMS. The formation of AP sites in genomic DNA. We are presently measuring the amount of endogenous AP sites in different species. To examine whether this assay can detect AP sites after cleavage of DNA 5' or 3' to an AP site, heat/acid-treated DNA was incubated with hAPE and/or NaOH at 37°C. There was no reduction in the number of AP sites in the DNA following 3' cleavage to the AP site by β-elimination with NaOH (19). In contrast, 5' incision by hAPE and both 5' and 3' nicks to AP sites by combination of hAPE and NaOH reduced the number of AP sites by 28 and 70%, respectively. The marked decrease in the number of AP sites appears to be due to the release of AP sites from the DNA back bone. The reason why hAPE treatment alone reduced the number of AP sites on the DNA may be due to the spontaneous induction of nicks 3' to AP sites during the incubation of DNA with heat/acid-solution. This experiment suggests that this assay may also be applicable to assessing AP endonuclease activity as well as determining the location of cleavage relative to AP sites.

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