Novel non-isotopic detection of MutY enzyme-recognized mismatches in DNA via ultrasensitive detection of aldehydes

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ABSTRACT

A highly sensitive method to detect traces of aldehyde-containing apurinic/apyrimidinic (AP) sites in nucleic acids has been developed. Based on this method, a novel approach to detect DNA base mismatches recognized by the mismatch repair glycosylase MutY is demonstrated. Open chain aldehydes generated in nucleic acids due to spontaneous depurination, DNA damage or base excision of mismatched adenine by MutY are covalently trapped by a new linker molecule [fluorescent aldehyde-reactive probe (FARP), a fluorescein-conjugated hydroxylamine derivative]. DNA containing AP sites is FARP-trapped, biotinylated and immobilized onto neutravidin-coated microplates. The number of FARP-trapped aldehydes is then determined via chemiluminescence using a cooled ICCD camera. AP sites induced in plasmid or genomic calf thymus DNA via mild depurination or by simple incubation at physiological conditions (pH 7, 37°C) presented a linear increase in chemiluminescence signal with time. The procedure developed, from a starting DNA material of ~100 ng, allows detection of attomole level (10^{-18} mol) AP sites, or 1 AP site/2×10^7 bases, and extends by 1–2 orders of magnitude the current limit in AP site detection. In order to detect MutY-recognized mismatches, nucleic acids are first treated with 5 mM hydroxylamine to remove traces of spontaneous aldehydes. Following MutY treatment and FARP-labeling, oligonucleotides engineered to have a centrally located A/G mismatch demonstrate a strong chemiluminescence signal. Similarly, single-stranded M13 DNA that forms mismatches via self-complementation (average of 3 mismatches over 7429 bases) and treated with MutY yields a signal ~100-fold above background. No signal was detected when DNA without mismatches was used. The current development allows sensitive, non-isotopic, high throughput screening of diverse nucleic acids for AP sites and mismatches in a microplate-based format.

INTRODUCTION

Screening of cells and human tissues for DNA damage is commonly conducted in order to correlate the action of clastogenic agents with human disease (1–6). Two aspects of DNA damage that have received considerable attention in recent years are the generation and measurement of apurinic/apyrimidinic (AP) sites and of mismatched DNA bases (7–17). Both lesions are considered promutagenic (7,17). AP sites occur spontaneously, as a result of DNA damage, or as an intermediate in the action of excision repair enzymes (18–21). When unrepaired, AP sites may direct polymerases to preferentially insert adenine in the opposite strand, thus promoting base substitution mutations (22). Base mismatches, on the other hand, occur as a result of errors in DNA replication by polymerases or by spontaneous chemical changes (e.g. deamination of methylcytosine to thymine; 17) or as a result of unrepaired oxidative DNA damage (23). The excision repair of mismatches by glycosylases generates AP sites as intermediates (7,8,17). Recent findings suggest a definite correlation between defects in the mismatch repair mechanism and cancer (24–26). The ability to sensitively and efficiently screen cells and tissues for AP sites and mismatches would be of major interest, as detection of these forms of DNA damage could have a direct correlation with clinical endpoints. In addition, most assays that can quantitate mismatches have also been eventually utilized for detection of point mutations (27–35), thereby allowing screening for another endpoint of major clinical significance.

Several methods for measuring AP sites have been reported, which generally allow the measurement of AP sites at the femtomole (10^{-15} mol; 10–13) or sub-femtomole (14) levels. Several of these methods require radioactivity (9,10,12). On the other hand, measurement of mismatches is a more difficult task, and assays used to detect DNA mismatches have had varying degrees of success (27–35). Assays that utilize mismatch cleavage (27,31) or mismatch repair (32,33) enzymes are

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increasingly used due to the associated convenience and specificity (34). A drawback of most enzymatic methods, however, is that normal DNA containing no mismatches is also recognized by the enzymes to some extent, thus posing limits to the technology (34). A mismatch repair enzyme that lacks any activity on normal DNA is MutY (35–37). Gel electrophoresis-based methods that utilize *Escherichia coli* MutY to detect mismatches and mutations reported highly selective activity towards mismatched adenine by this enzyme (38). However, the use of gel electrophoresis poses restrictions as to the size and composition of DNA sequences that can be examined at a time, and cannot be used for genome-wide scanning of mismatches or for examining numerous samples at once. In addition, it can be laborious and it requires radioactivity.

The present work reports on a novel, non-isotopic methodology to detect AP sites or MutY-recognized mismatches in genomic DNA, based on the trapping of associated aldehydes by a new fluorescent aldehyde-reactive probe (FARP) (Fig. 1). FARP is a fluoresceine-conjugated derivative of hydroxylamine that forms stable oxime bonds with aldehydes and is unreactive towards normal DNA structures and 3′ OH ends (39). Both DNA depurination or excision repair of adenine in DNA by MutY result in open chain aldehydes (17). We describe the development of a new chemiluminescence methodology (diagrammatically shown in Fig. 1) to detect FARP-trapped aldehyde-containing AP sites on DNA. Subsequently, we demonstrate that MutY-recognized mismatches in DNA may also be detected with high sensitivity and specificity following FARP labeling and chemiluminescence measurement. The new methodology for detection of AP sites and MutY-recognized mismatches is performed on a microplate format, and small (~50 bp) as well as large (>1000 bp) DNA segments can be conveniently and rapidly examined, either for AP sites or for MutY-recognized mismatches, depending on the desired endpoint.

MATERIALS AND METHODS

DNA, oligomers and chemicals

FARP [5-(((2-(carbohydrazino)-methyl) thio) acetyl)-amino-fluorescein, aminooxyacetyl hydrazide] was synthesized as described (39). High purity genomic calf thymus DNA and double-stranded ladder (pUC18 *Msp*I digest, 26–501 bp) was purchased from Sigma Chemical and used without further purification. Single-stranded (+strand) M13 DNA was purchased from Pharmacia Biotech and pGXIs14 plasmid DNA, a gift from Professor MacLeod (MD Anderson Cancer Center) was isolated from the host bacteria as described earlier (39). Both agarose gel electrophoresis and the absorbance ratio at 260–280 nm were performed to determine the purity of the plasmid. Gel-purified 49mer oligonucleotides representing the TFIIIA transcription factor-binding sequence of the *Xenopus* rRNA gene (enumerated in Table 1) were supplied by Oligos Etc Inc. Enzyme MutY (*E.coli*) was purchased from Trevigen Inc. and stored as recommended by the manufacturers. Hydroxylamine purchased from Sigma Chemical was always freshly made prior to the experiments. GTG agarose was obtained from FMC Bioproducts, polyacrylamide gel electrophoresis reagents were from National Diagnostics while SYBR GOLD nucleic acid gel stain and Picogreen™ DNA quantitation dye was supplied by Molecular Probes. For chemiluminescence studies, Reacti-Bind NeutrAvidin coated polystyrene plates (pre-blocked with bovine serum

albumin) were supplied by Pierce. Anti-fluorescein-Fab fragments (Sheep)-alkaline phosphatase conjugate (antiF-Fab-AP) was purchased from Boehringer Mannheim. CDP-Star, a 1,2-dioxetane chemiluminescent enzyme substrate and Emerald-II™ enhancer used with CDP-Star was purchased from TROPIX Inc. Micro Bio-Spin G25 chromatography columns were obtained from Bio-Rad laboratories. Label IT™ Nucleic Acid biotinylation kit was purchased from PanVera Inc. All reagents and buffers were of analytical grade and made with ultrapure water (1800 Mohm m⁻¹ resistivity) delivered by an Alpha-Q system (Millipore).

Acidic or physiological depurination of calf thymus DNA: treatment with hydroxylamine

Aldehyde-containing AP sites were chemically induced in calf thymus or plasmid DNA by a short exposure (0–60 s) to acidic conditions (pH 3.5) over a set time period at a temperature of 38°C, as described earlier (39). The reaction was halted by placing the sample quickly on ice and adding a neutralization solution (10% of 3 M sodium acetate and 1 M potassium phosphate buffer at pH 7 and 7.5, respectively), to a final volume of 50 μl. AP sites were also slowly generated in calf thymus DNA via spontaneous depurination at 37°C, pH 7.0, over a period of days, and these were monitored with the present assay. Prior to incubation at 37°C, the DNA was treated with 5 mM hydroxylamine for 1 h at room temperature to remove traces of existing aldehydes from the pool of potential FARP-binding sites. The hydroxylamine was then removed via G25 ultracentrifugation and the sample was resuspended in sodium phosphate buffer, pH 7.

DETECTION OF ALDEHYDE-containing AP SITES AND MutY - RECOGNIZED MISMATCHES IN DNA

FARP

1. DETECTION OF AP SITES
APURINIC/APYRIMIDINIC SITE

2. DETECTION OF MutY-recognized MISMATCHES (e.g. A/G)

REMOVE SPONTANEOUS AP SITES (HYDROXYLAMINE TREATMENT)

TREAT w. MutY LABEL RESULTING ALDEHYDES W. FARP

BIOTINYLATE DNA

IMMOBILIZE FARP - LABELED DNA ON NEUTRAVIDIN MICROPLATES
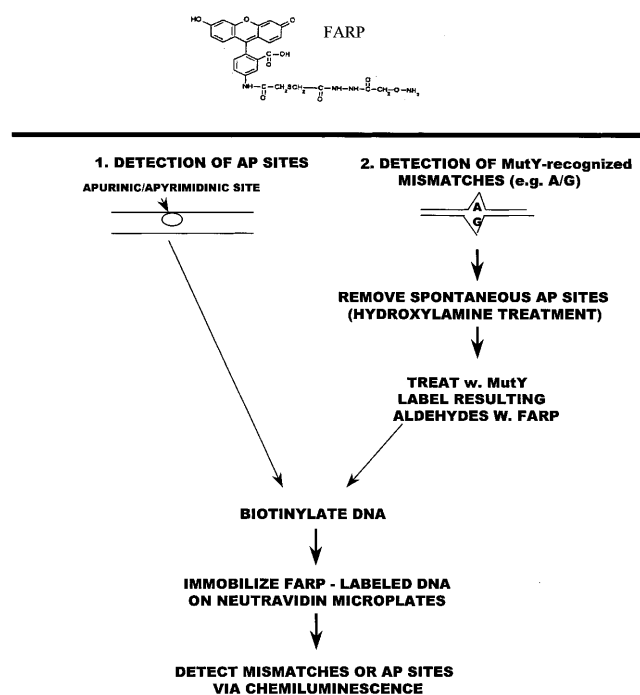
DETECT MISMATCHES OR AP SITES VIA CHEMILUMINESCENCE

Figure 1. Flow chart of the current approach for detection of AP sites or MutY-recognized mismatches in DNA. The structure of FARP is also shown.

FARP trapping of aldehydes and subsequent DNA biotinylation

To covalently trap open-chain aldehydes generated in DNA at the position of AP sites, 500 µM FARP was reacted with ~100 ng DNA in 40 mM sodium citrate pH 7.0 at 15–22°C for 30 min. In some experiments, FARP labeling of DNA was performed simultaneously with the depurination reaction. Non-covalently bound FARP was removed by G25 ultracentrifugation. FARP-labeled DNA was either used on the same day or stored at 4 or –20°C for a few days prior to further experiments. To immobilize FARP-labeled DNA onto neutravidin microplates, the DNA was exposed for 1 h to a commercially available biotinylation reagent (Biotin Label IT™ reagent, 1 µl reagent/µg DNA, in MOPS buffer, pH 7.5, at 37°C). Excess reagent was then removed by G25 ultracentrifugation. The samples were either used immediately or stored at 4°C for a few days, prior to chemiluminescent studies.

Chemiluminescence measurement of FARP-trapped aldehydes in calf thymus or plasmid DNA

Double-stranded DNA, doubly-labeled with FARP and biotin, was immobilized onto neutravidin-coated microplate strips, in the presence of 5 nM antiF-Fab-AP. In a typical experiment, 50–100 ng of doubly-labeled DNA plus 5 nM antiF-Fab-AP in a total volume of 50 µl were incubated at room temperature for 1 h in TE buffer, pH 7.5. Unbound sample and antiF-Fab-AP were removed by pipetting and washing with TE at least four times. The microplate strips were then transfered into 50 ml polypropylene tubes and washed four times in 30–50 ml of TE buffer with constant agitation for 10 min. The chemiluminescent substrates (CDP-Star plus Emerald II enhancer) were then added in 0.1 M diethanolamine, pH 8.5, and the antiF-Fab-AP-catalyzed dephosphorylation of CDP-Star reaction was carried out at room temperature for 1 h, after which maximum light generation was achieved. In separate experiments to quantitate the fraction of biotinylated DNA captured on microplates, Picogreen™ dye was used to measure double-stranded DNA just prior and after its removal from neutravidin-coated plates.

Chemiluminescence instrumentation

The low light from the chemiluminescence reaction was detected using an intensified charged coupled device (ICCD) system (Princeton Instruments). This ICCD camera utilizes a proximity focused microchannel plate (MCP) image intensifier, fiber-optically coupled to the CCD array. The entire area of the ICCD is capable of light detection, giving a total of 576×384 pixels on a Pentium® PC computer screen. Both the intensifier and CCD are cooled to –35°C thermoelectrically and the dark current is <50 c.p.m. The ICCD was used to detect total light generation from each cell of the microplate strip. Cells were individually placed in a reproducible geometry at short distance from the ICCD and the total light output per second measured. The background chemiluminescence (signal measured when FARP was omitted from the procedure) was routinely subtracted from all samples. All measurements were repeated at least three times.

Formation of homoduplex and heteroduplex oligonucleotides

49mer oligonucleotides and their complementary strands with or without a centrally located T-to-G base substitution were synthesized. In another synthesis of the same oligomers, 5′ biotinylated 49mers and their complementary unbiotinylated strands were synthesized (Table 1). For hybridization, equimolar amounts (~0.5 µg) of each oligonucleotide were annealed in 40 mM Tris–HCl (pH 7.5), 20 mM MgCl₂ and 50 mM NaCl to form duplex oligonucleotides. The mixture was first heated to 95°C for 2 min, then allowed to hybridize at 65°C for 3 h and cooled slowly to room temperature. Following hybridization, the double-stranded 49mers were treated with hydroxylamine (5 mM in citrate pH 7.0, for 30 min, 25°C) to remove traces of spontaneously or heat-generated aldehydes from the pool of FARP-reactive sites.

Treatment of M13 DNA, ladder DNA and duplex oligonucleotides with MutY and gel electrophoresis

Test DNA (single-stranded M13, ladder DNA or duplex oligonucleotide) (50 ng) was incubated for 1 h at 37°C with 0–1.0 U MutY in 40 mM Na-citrate buffer (pH 7.0) and then alkali-treated to convert positions of missing adenines to strand breaks. Analysis of cleavage products for single-stranded M13 DNA was done by agarose gel electrophoresis (0.9% agarose, run overnight at 20 V in 1× TBE buffer and stained with 1 µg/ml ethidium bromide). Fragment analysis for ladder DNA and oligonucleotides was done by 16% denaturing polyacrylamide gel electrophoresis in the presence of 7.5 M urea at 20 V/cm. The DNA fragments were detected by SYBR Gold dye or by ethidium staining and photographs taken using an Eagle Eye™ Still Video (Stratagene).

Chemiluminescence measurement of FARP-trapped mismatches in oligonucleotides, ladder and M13 DNA

M13 DNA, ladder DNA or 5′-biotinylated oligonucleotide duplexes, hydroxylamine treated, were exposed to MutY, FARP labeled, and biotinylated with the protocols described above. The biotinylation step was omitted for the oligonucleotides since these were pre-biotinylated. Typically, 50 ng from the doubly (biotin plus FARP)-labeled nucleic acids were applied on neutravidin-coated microplates and their chemiluminescence measured.

Table 1. Sequences of the synthesized oligonucletides

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1. B-5′-GTC TCC CAT CCA AGT ACT AAC CAG GCC CGA CCC TGC TTG GCT TCC GAT T-3′

2. B-5′-AAT CGG AAG CCA AGC AGG GTA GGG CCT GGT TAG TAC TTG GAT GGG AGA C-3′

3. B-5′-AAT CGG AAG CCA AGC AGG GTA GGG CCT GG**G** TAG TAC TTG GAT GGG AGA C-3′

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1 and 2 are complementary and form a homoduplex. 1 and 3 form a heteroduplex with an A/G mismatch at position 20. On a separate set of oligonucleotides, a biotin molecule (B) was incorporated at the 5′ end during synthesis.
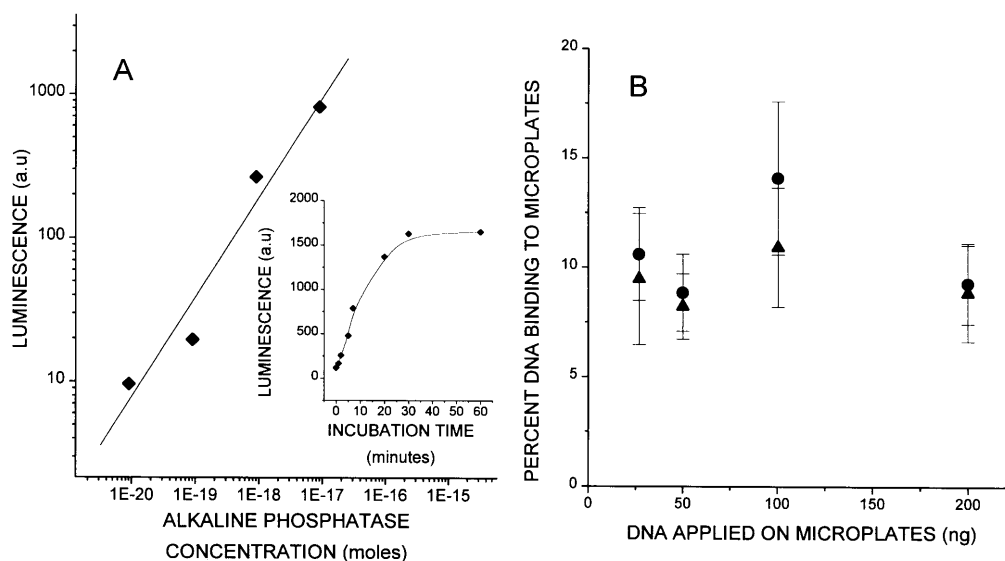
Figure 2. (A) Sensitivity of chemiluminescence detection of alkaline phosphatase with the present cooled ICCD camera and chemiluminescent substrates. Inset shows buildup of chemiluminescence following addition of substrate plus enhancer. (**B**) Percent DNA binding to microplates, as a function of amount nucleic acid applied. Triangle, 49mer oligonucleotides; circle, calf thymus DNA.

RESULTS

Dual labeling of DNA and chemiluminescence detection using the present protocol

Figure 2A shows chemiluminescence obtained with the present setup when serial dilutions of free alkaline phosphatase were added to CDP-STAR® substrate and Emerald II enhancer and measured using the cooled ICCD. The chemiluminescence detection limit of this setup is <0.01 amol alkaline phosphatase. Examination of the buildup of alkaline phosphatase chemiluminescent signal in solution following addition of substrate plus enhancer at room temperature demonstrates that after 60 min a relatively constant value is achieved (Fig. 2A, inset). Therefore, all measurements reported were conducted 60–80 min following addition of the substrate. To estimate the fraction of biotinylated DNA captured on the neutravidin-coated microplates, biotinylated nucleic acid was quantitated using the fluorescence of Picogreen™ dye prior to its application and immediately following complete removal of unbound DNA from microplates. Oligonucleotides and calf thymus DNA yielded $(8 \pm 2)\%$ and $(11 \pm 3)\%$ DNA binding on the plates, respectively (Fig. 2B). An average of ~10% DNA binding on the plates was therefore assumed for all subsequent calculations.

Ultrasensitive detection of aldehydes in DNA

Chemiluminescence detection of aldehyde-containing AP sites generated in 100 ng plasmid DNA following depurination in sodium citrate, pH 3.5, at 38°C for up to 60 s and trapping of AP sites by FARP is depicted in Figure 3. The induction of luminescence is linear with respect to depurination exposure. The curve has been forced through zero by subtracting from all points the luminescence at zero depurination. The inset, from an earlier work (39), demonstrates detection of fluorescence following FARP-labeling of this same plasmid exposed under identical conditions to higher depurination times (0–60 min). The fluorescence-based

approach is less sensitive than the present method; however, it allows direct quantitation of the number of FARP molecules per DNA base pair, as well as the absolute number of AP sites measured (39). Five minutes depurination of 1 µg DNA resulted in fluorescence originating from 88.2 fmol AP sites, or 1 AP site/34 000 bases (39). Assuming a linear decrease of AP sites for lower depurination exposures, the 15 s exposure in Figure 3 corresponds to ~1 AP site/7×10^5 bases. Considering also the data of Figure 2B, the amount of microplate-captured DNA generating the chemiluminescence signal for 15 s depurination is ~10 ng. Therefore, the absolute number of AP sites recorded following 15 s depurination is ~43 amol (see right axis in Fig. 3).

To estimate the lowest number AP sites detectable, hydroxyl-amine treatment of genomic calf thymus DNA was first employed to remove traces of pre-existing AP sites [e.g. AP sites expected to be present (14,19) in genomic DNA from mammalian cells prior to DNA extraction, plus AP sites generated—or removed—during handling]. Hydroxylamine is a small molecule and is expected to react rapidly with aldehydes, as previously demonstrated for methoxyamine (10), thereby prohibiting sub-sequently added FARP to react at the same positions. Figure 4A depicts the decrease in the chemiluminescence signal obtained following hydroxylamine treatment of genomic calf thymus DNA undepurinated, or depurinated for 15 s. Following hydroxyl-amine removal and reaction with FARP, the chemiluminescence was reduced to almost background levels (~500 U luminescence), which defines the current limit of this method. According to Figure 3, 500 U luminescence correspond to ~2 amol AP sites, or 1 AP site/2×10^7 bases, using a starting DNA material of 100 ng of which ~10 ng are captured on the microplate. When hydroxylamine-treated calf thymus DNA was kept at 37°C, phosphate buffer pH 7, and assayed for AP sites via FARP as a function of time, a linear increase in spontaneously-generated aldehydic AP sites (3–425 amol) was detected (Fig. 4B). DNA kept at 4°C under similar conditions did not display any increase in luminescence signal (Fig. 4B).
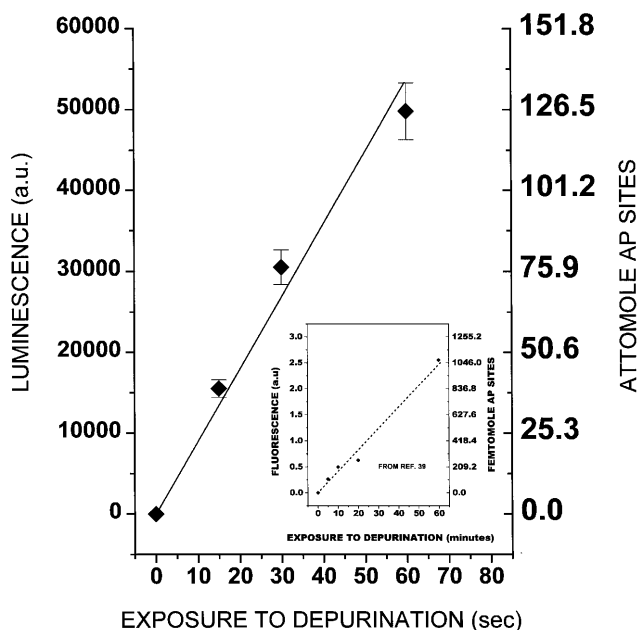
Figure 3. Chemiluminescence detection of aldehyde-containing AP sites generated in plasmid DNA following depurination in sodium citrate, pH 3.5, at 38°C for up to 60 s. The luminescence at zero value has been subtracted. Inset depicts fluorescence detection when extensive depurination under identical conditions is applied. Data in the inset were used to relate depurination times to AP sites (right axis, see text).

Gel electrophoresis of MutY-treated oligonucleotides and single-stranded M13 DNA

49mer oligomers engineered to form a double-stranded structure, with or without a centrally located A/G mismatch upon hybridization, were exposed to MutY, alkali treated and examined upon denaturing gel electrophoresis. Generation of the two expected fragments was observed for the heteroduplex oligomers, while no cutting was present in the homoduplexes (Fig. 5A). Under the conditions applied, the fragmented DNA was ~30% of the total DNA per lane, as quantified by a microdensitometer. This is significantly <50% of the total DNA per lane, which would result

if all A/G mismatches were acted upon by MutY. The homoduplex-containing double-stranded DNA ladder (26–501 bp fragments) did not demonstrate additional fragmentation following enzymatic treatment (Fig. 5B). In contrast, MutY treatment of the 7249 base-long M13 single-stranded DNA resulted, presumably via generation of mismatches by self-complementation, to the generation of approximately six fragments, the smallest of which is ~1000 bases long, as demonstrated in Figure 5C. Figure 5C also demonstrates that MutY has no activity on double-stranded plasmid, neither in the presence nor absence of single-stranded M13 (lanes 3–5). If it is assumed that each enzymatically recognized site is cut with <100% efficiency by MutY, then three DNA fragments occur per cut site: the two DNA fragments plus the original one. Therefore, from Figure 5C, lane 2, it can be inferred that, to generate six discrete fragments, an average of about three MutY-recognized cutting sites are generated per each 7249 base-long M13 molecule.

FARP-based chemiluminescence detection of mismatches in high and low molecular weight DNA

Starting with 100 ng of biotinylated 49mer homoduplexes or heteroduplexes, the nucleic acid was treated successively with hydroxylamine, MutY, then FARP, and applied on neutravidin microplates for chemiluminescence detection of mismatches. A strong signal was obtained for A/G mismatch-containing oligonucleotides (Fig. 6), corresponding to 49mers simultaneously bound by the immobilized neutravidin and by the antiF-Fab-AP. No signal was obtained when MutY was omitted, or when oligonucleotides without mismatch were MutY treated. A mixture of double-stranded homoduplexes (DNA ladder, presumed to contain no mismatches) treated in the same way also demonstrated absence of chemiluminescence signals (Fig. 7). In contrast, single-stranded M13 demonstrated a chemiluminescence signal of about 100 times the signal obtained without MutY, indicating the generation of FARP-reactive sites following MutY treatment (Fig. 7). The chemiluminescence results agree with the fragmentation results obtained by gel electrophoresis (Fig. 5). Since MutY possesses an associated lyase activity (23,26; Trevigen Inc., personal communication), it is possible that labeling of MutY-digested M13 DNA with FARP occurs at positions of the generated 3′ aldehydes, as well as AP-aldehydes.
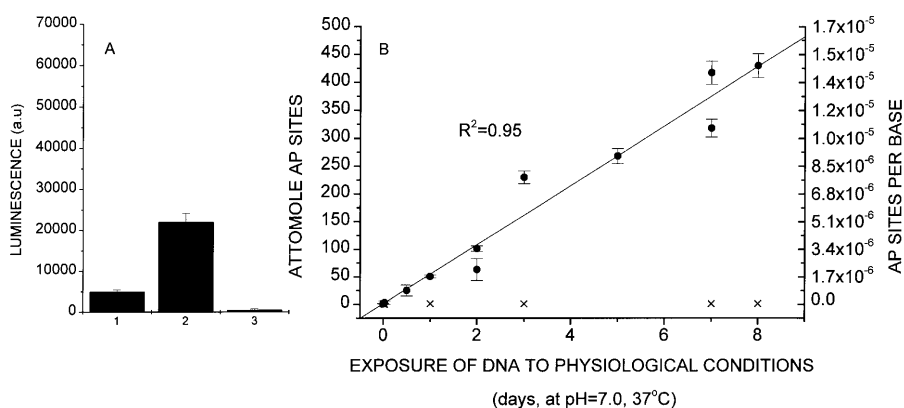

Figure 4. (**A**) Detection of AP sites in genomic calf thymus DNA without hydroxylamine treatment (bar 1, undepurinated DNA; bar 2, DNA depurinated for 15 s), or following treatment with hydroxylamine (bar 3). (**B**) Detection of spontaneously generated AP sites in hydroxylamine-treated DNA at pH 7.0, at a temperature of 37°C (circle) or 4°C (cross). Error bars represent standard deviation of three to five experiments.
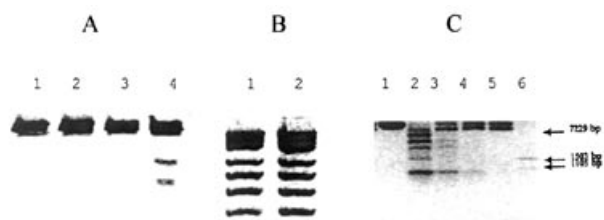
Figure 5. Gel electrophoresis of MutY-treated DNA, examined on denaturing gels. (**A**) 49mer double-stranded oligonucleotides are MutY-treated and visualized on polyacrylamide gels following SYBR GOLD staining. Lane 1, no mismatch, no MutY; lane 2, no mismatch, plus MutY; lane 3, A/G mismatch, no MutY; lane 4, A/G mismatch, plus MutY. (**B**) Double-stranded homoduplex mixtures (DNA ladder, 26–501 bp) are MutY treated and visualized on polyacrylamide gels following SYBR GOLD staining. Lane 1, no MutY; lane 2, plus MutY. (**C**) Single-stranded M13 DNA (7249 bases) are enzymatically treated and visualized on agarose gels following ethidium staining. Lane 1, M13 DNA, no MutY; lane 2, M13 DNA, plus 1 U MutY; lane 3, double-stranded plasmid (90%), plus M13 DNA (10%), plus 1 U MutY; lane 4, double-stranded plasmid (99%), plus M13 DNA (1%), plus 1 U MutY; lane 5, double-stranded plasmid (100%), plus 1 U MutY; lane 6, molecular weight marker.
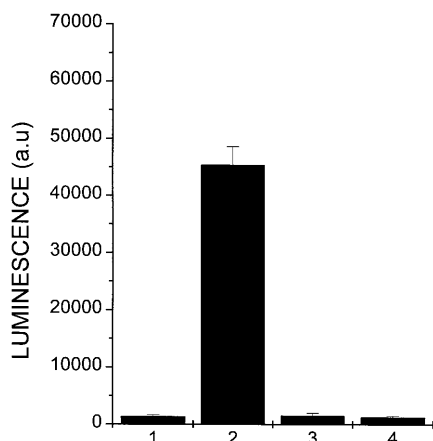

Figure 6. Chemiluminescence detection of MutY-treated oligonucleotides: 49mers are enzymatically treated, FARP labeled and captured on microplates. Bar 1, A/G mismatch, no MutY; bar 2, A/G mismatch, plus MutY; bar 3, no mismatch, no MutY; bar 4, no mismatch, plus MutY. Results are expressed as average of three experiments ±SD.

DISCUSSION

Both AP sites and base mismatches in DNA can lead to mutagenesis (7,17). In an attempt to develop methods sensitive enough to perform AP site detection and mismatch scanning in cells and tissues, we utilized a recently developed FARP (39) to quantitate AP sites via chemiluminescence. Unlike hydrazine (-NH-NH$_2$)-based probes, hydroxylamine (-O-NH$_2$)-based compounds such as FARP react very specifically with aldehydes and show no detectable cross-reactivity with other DNA components (10,13,39). Previous quantitations of AP sites have demonstrated femtomole (10^{-15} mol) level sensitivity (9–13). In the most sensitive AP site detection method reported, Nakamura *et al.* (14), using a biotin probe, detected ~1 AP site/4×10^6 bases in a slot-blot based protocol requiring a starting DNA material of 15 μg. In the current approach, starting from 100 ng DNA, attomole
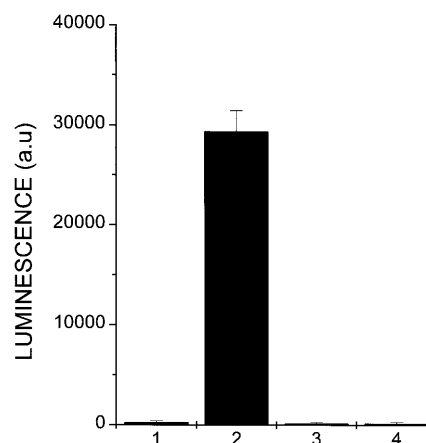

Figure 7. Chemiluminescence detection of MutY-treated DNA fragments of varying length: single-stranded M13 DNA (7249 bases) and double-stranded homoduplex mixtures (DNA ladder, 26–501 bp) are enzymatically treated, FARP labeled and captured on microplates. Bar 1, M13 DNA, no MutY; bar 2, M13 DNA, plus MutY; bar 3, ladder DNA, no MutY; bar 4, ladder DNA, plus MutY. Results are expressed as an average of three experiments ±SD.

(10^{-18} mol) level detection of AP sites is obtained, and ~1 AP site/2×10^7 bases is detected. Since the overall sensitivity of any method is directly related to the total DNA that can be introduced into a particular assay system (40), the ability to perform the assay with nanogram-amount DNA is very significant. Therefore, depending on the chosen criterion, the current microplate-based method extends the limits of AP detection by 1–2 orders of magnitude. By using FARP to quantitate spontaneously generated AP sites in double-stranded genomic calf thymus DNA at physiological conditions, it was derived that ~1.7 AP sites/10^6 nucleotides/day are generated (Fig. 4B). This translates to 10 200 AP sites generated/day/mammalian genome (~6×10^9 bases) and is in agreement with calculated (10 000 AP sites/cell/day; 7) and measured (9000 AP sites/cell/day; 14) values.

During excision repair of dA:dG, dA:oxodG and to a lesser extent dA:dC mismatches, the *E.coli* MutY glycosylase removes the adenine via complex chemistry (23,35–37) which generates an aldehyde on the sugar (17). We took advantage of this fact to covalently trap positions of mismatched adenine with FARP, and subsequently detect it with a sensitive chemiluminescence procedure. Unlike other mismatch-recognizing enzymes (27,31,33), MutY has no reported glycosylase activity on normal DNA (23,35–38), thereby allowing highly specific recognition of mismatches. In agreement to these expectations, the data in Figures 5–7 demonstrate that the action of MutY on DNA is a necessary and sufficient condition for generation of chemiluminescence signals. Using the present method it is possible to screen diverse and large DNA fragments for MutY-recognized mismatches in a single experiment, something practically unachievable by gel electrophoresis or current mismatch detection methods. In addition, base substitution mutations can be measured by technologies detecting mismatches (27–35). Thereby the possibility opens up for high-throughput screening of another endpoint of major clinical significance using the present approach.

In conclusion, a highly sensitive method to detect aldehyde-containing AP sites in DNA was developed, on the basis of which

a technique to perform mismatch scanning in long and diverse DNA fragments was devised. The amount of starting nucleic acid required for the assay is small (~100 ng), and the application is simple and can be automated to analyze several samples simultaneously on microplates. It is anticipated that this technology may be used to sensitively scan diverse genes in cells and tissues for AP sites, mismatches and potentially base substitution mutations.

ACKNOWLEDGEMENTS

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