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A molecular beacon assay for measuring base excision repair activities

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Abstract

The base excision repair (BER) pathway plays a key role in protecting the genome from endogenous DNA damage. Current methods to measure BER activities are indirect and cumbersome. Here, we introduce a direct method to assay DNA excision repair that is suitable for automation and industrial use, based on the fluorescence quenching mechanism of molecular beacons. We designed a single-stranded DNA oligonucleotide labelled with a 5′-fluorescein (F) and a 3′-Dabcyl (D) in which the fluorophore, F, is held in close proximity to the quencher, D, by the stem–loop structure design of the oligonucleotide. Following removal of the modified base or incision of the oligonucleotide, the fluorophore is separated from the quencher and fluorescence can be detected as a function of time. Several modified beacons have been used to validate the assay on both cell-free extracts and purified proteins. We have further developed the method to analyze BER in cultured cells. As described, the molecular beacon-based assay can be applied to all DNA modifications processed by DNA excision/incision repair pathways. Possible applications of the assay are discussed, including high-throughput real-time DNA repair measurements both in vitro and in living cells.

Keywords: Molecular beacon; DNA glycosylase; AP endonuclease; Base excision repair; Nucleotide incision repair; Oxidative DNA damage

Reactive oxygen species-induced non-bulky DNA damage is believed to contribute significantly to cell lethality, tissue degeneration, ageing, and cancer [1]. To counteract the deleterious effects of these lesions, which may lead to genomic instability, cells have evolved a number of DNA repair mechanisms, including the base excision repair (BER) pathway [2]. In BER, DNA glycosylases recognize and remove damaged and/or mispaired bases from DNA by cleavage of the *N*-glycosylic bond between the abnormal base and deoxyribose, leaving either an abasic site or a single-strand nick in DNA [3]. As the biological consequences of persisting endogenously and exogenously generated DNA modifications, together with variations in DNA repair activities are connected to both individual and population disease susceptibility [4,5], the development of rapid and high-throughput screening assays for BER activities is of fundamental importance. Current assays for DNA glycosylase and AP endonuclease activities are time-consuming and indirect since they are based on separation techniques such as solubility in acid and/or ethanol [6], chromatography [7], gel electrophoresis [8], paramagnetic beads [9], etc. Generally, DNA substrates are radioactively labelled to measure BER activity in a quantitative manner, although non-radioactive, fluorometric oligonucleotide assays coupled to gel electrophoresis have also been described [10]. Importantly, the requirement for an additional step to separate the reaction product precludes

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the use of existing techniques for real-time measurements and monitoring BER in living cells and makes them difficult to automate to enable large scale experiments to be undertaken. In an attempt to overcome these difficulties, Stivers [11] reported a 4- to 8-fold enhancement in fluorescence upon formation of an abasic site adjacent to, or opposite, a 2-aminopurine residue in the substrate DNA. Based on this result, a continuous 2-aminopurine fluorescence assay for *Escherichia coli* uracil–DNA glycosylase (UNG) activity was developed [11,12]. However, this approach was limited to *E. coli* UNG and was not extended to other DNA repair enzymes.

As first described, a molecular beacon is a single-stranded oligonucleotide probe containing a sequence complementary to a target that is flanked by self-complementary termini, and carries a fluorophore and a quencher at the 5′- and 3′-ends [13]. When used as a hybridization probe in the absence of the DNA target, these molecules form a stem–loop structure in which the 5′-fluorophore and 3′-quencher end up in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by fluorescence resonance energy transfer [14]. Subsequent binding to the target or scission of the stem–loop structure leads to separation of the fluorophore-quencher pair and a resulting increase in fluorescence. This property has led molecular beacons to be used for various applications in addition to the detection of DNA and RNA targets, including assays to follow the activity of single-strand specific DNases [15], the restriction endonuclease *Bam*HI [16], small non-enzyme DNA cleavage agents [17], and ribonuclease H [18]. Here, we report for the first time a direct DNA repair assay based on molecular beacon oligonucleotides containing modified residues that allows direct and high-throughput measurements to be obtained both in vitro and in living cells.

Materials and methods

Oligonucleotides. All oligodeoxyribonucleotides were purchased from Eurogentec (Seraing, Belgium) including the following oligonucleotides:

non-fluorescent stem–loop oligonucleotides,

- SL-UDG, d(GCACUUAAGAAUUCACGCCATGTCGAAAUUCUUAAGUGC);
- SL-APE1, d(GCACTUAAGAATTCACGCCATGTCGAAATTCTTAAGTGC), where U is uridine;

molecular beacons,

- FL-35, (FITC)-d(GCACTTAAGAATTCACGCCATGTCGAAATTCTTAAGTGC);
- FD-35, (FITC)-d(GCACTTAAGAATTCACGCCATGTCGAAATTCTTAAGTGC)-Dabcyl, where FITC is fluorescein isothiocyanate and Dabcyl is 4-(4′-dimethylaminophenylazo)benzoic acid;

modified molecular beacons,

- FD-UDG, (FITC)-d(GCACUUAAGAAUUCACGCCATGTCGAAAUUCUUAAGUGC)-Dabcyl;
- FD-APE1 and FD-THF, (FITC)-d(GCACT**X**AAGAATTCACGCCATGTCGAAATTCTTAAGTGC)-Dabcyl, where X is a uridine or a tetrahydrofuran residue, respectively;
- FD-INO, (FITC)-d(CGC**I**TC**I**AC**I**C**I**T-$(CH_2)_{18}$-AC**I**C**I**TC**I**AC**I**CG)-Dabcyl, where I is an inosine; and
- FD-8oxoG · A, (FITC)-d(GCACT**X**AAGAATTCACGCCATGTCGAAATTCTTAAGTGC)-Dabcyl, where X is 7,8-dihydro-8-oxo-2′-deoxyguanosine.

These sequence contexts were previously designed as anti-sense probes for RNA targets [19]. The secondary structure of the beacons was calculated using RNAStructure 3.5 software. All the oligonucleotides can form a stem–loop structure with 13 nucleotides situated in the loop and 13 bp in the stem. To ensure that the oligonucleotides correctly adopted their hairpin structure, they were heated at 95 °C for 3 min and slowly cooled to room temperature and the secondary structure was confirmed by melting experiments. End labelling and preparation of reduced AP sites were performed as described [20].

Cell lines and culture. *Saccharomyces cerevisiae* strains isogenic to MKp-o (MATa, *leu*2-3,112, *his*3-*Δ* 1, *trp*1-289am, *ura*3-52, and *gal*2), DRY373 (*apn1Δ*::*his*3), RBY31 (*apn2Δ*::*his*G), RBY71 (*apn1Δ*::*his*3, *apn2Δ*::*his*G) were kindly provided by Dr. D. Ramotar (University of Montreal, Canada). HeLa and NIH3T3 cells were grown in minimum essential medium and Dulbecco's modified Eagle's medium (Gibco), respectively, at 37 °C in a humidified atmosphere with 5% CO_2. Culture media were supplemented with 10% of heat inactivated fetal calf serum (Gibco), 100 mg/mL streptomycin, and 100 U/mL penicillin. *S. cerevisiae*, HeLa, and mouse embryonic fibroblast (MEF) whole cell-free extracts were prepared as described [20].

Enzymes and inhibitors. The hUNG recombinant protein lacking the N-terminal 84 amino acids (27 kDa, 230 aa) was purified as described [21]. Purification of human apurinic/apyrimidinic endonuclease (APE1) was performed as described [20]. Uracil–DNA glycosylase inhibitor (UGI) was purchased from New England BioLabs (OZYME, Saint Quentin Yvelines, France). The spontaneous heat denatured product (HDP) of the oligonucleotide containing an abasic site was prepared as described [22]. Briefly, SL-APE1 was treated with hUNG and the reaction products were heated at 95 °C for 15 min then cooled at room temperature for 5 min, and kept on ice until use. The resulting HDP was used for the inhibition of APE1 activity.

Enzyme assays. The standard enzyme assay with 5′-^{32}P-labelled oligonucleotides was performed as described [20]. Reaction products were analyzed by electrophoresis in 10% (w/v) non-denaturing polyacrylamide gels (29:1 acrylamide/bisacrylamide) containing 0.5× TBE, at 100 V for 15 h at 20 °C. Gels were then exposed to a Storm 840 Phosphor Screen and the amount of radioactivity in the bands was quantified using ImageQuaNT software. The standard enzyme assay with molecular beacons was performed at room temperature with 5–10 nM of modified beacons and either pure protein or cell-free extract, in the respective reaction buffer, unless otherwise stated. Reactions were performed in a quartz cuvette (final volume 0.4 mL) and fluorescence was measured using an SFM 25 Kontron fluorimeter and real-time computed with the attached software WIND25 1.50. Excitation was at 488 nm and emission at 515 nm with fluorescence expressed as response units (RU). At the end of the reaction, in order to compare the fluorescence of the reaction product with that of the reaction mixture without enzyme, emission spectra of the reaction product, and the reaction mixture without enzyme were obtained between 500 and 550 nm and with an excitation at 488 nm.

Repair assay in living cells. HeLa (2×10^5) and NIH3T3 (1.5×10^5) cells were grown in 6-well plates. Cells were washed twice with PBS and the culture medium was replaced with 0.9 ml of OptiMEM medium (Gibco) before transfection. Two hundred picomoles of each oligonucleotide and 2 μg of Cytofectin GSV (Glen Research, USA) were diluted separately in 50 μl of solution A (100 mM NaCl, 10 mM Hepes, pH 7.4), and then mixed together. After 15 min incubation at room temperature, Cytofectin · DNA complexes were added drop by drop to the cells and incubated for 5 h at 37 °C. Transfected cells were then fixed with 4% formaldehyde in PBS for 20 min at 4 °C. Formaldehyde was removed by washing once with PBS supplemented with 50 mM NH_4Cl and twice with PBS. Fluorescent images of living cells

in PBS were obtained using a fluorescence microscope (Axioskop, Zeiss) and documented using a digital camera and Adobe Photoshop software (Moutain View, CA).

Results and discussion

To develop a BER assay using molecular beacon technology, we used human uracil–DNA glycosylase (hUNG) and APE1. The hUNG protein specifically excises uracil from single- and double-stranded DNA leaving abasic (AP) sites [23], while the APE1 protein incises the phosphodiester backbone 5′ to the AP site and plays a key role in BER [24]. Thus, we designed the stem–loop forming single-stranded oligonucleotides SL-UDG and SL-APE1, containing either multiple U · A base pairs or a reduced AP site, respectively. To generate a stable AP site in SL-APE1, the oligonucleotide containing a single uracil residue at position six was treated with hUNG and then reduced with $NaBH_4$. Cleavage of the AP site generates a 5mer fragment which does not anneal to the rest of SL-APE1 at room temperature. To verify the conformational changes of the 5′-^{32}P-labelled SL-UDG and SL-APE1 induced by hUNG and APE1, respectively, DNA duplex melting was monitored by non-denaturing polyacrylamide gel electrophoresis (PAGE). As expected, hUNG and APE1 induce melting of the stem–loop oligonucleotide under non-denaturing conditions (Fig. 1). The DNA sequences of SL-UDG and SL-APE1 were then used to design the following molecular beacons carrying a 5′-fluorophore (FITC) and 3′-quencher (Dabcyl): FD-UDG, FD-APE1, and FD-35, a control DNA probe in which all uracil residues were replaced by thymine. In a typical molecular beacon, the quenching efficiency of the FITC–Dabcyl pair is about 99.9% [25]. Controlled heat denaturation of the modified molecular beacon resulted in a fluorescence signal enhancement of up to 40-fold (data not shown). As shown in Fig. 2, incubation of hUNG with FD-UDG (A) and APE1 with FD-APE1 (B) (pre-treated by hUNG/$NaBH_4$) induced a time-dependent increase in fluorescence. No fluorescence was observed when FD-35 was used indicating the absence of non-specific DNA degradation and/or denaturation (Figs. 2A and B). To test the specificity of the beacons we used the uracil–DNA glycosylase inhibitor (UGI) encoded by the *Bacillus subtilis* bacteriophage PBS [26] and a heat degradation product (HDP) as an APE1-inhibitor [22]. As expected, in the presence of the appropriate inhibitor the increase in fluorescence is markedly reduced (Figs. 2A and B), indicating the specificity of hUNG and APE1 for the beacons, and illustrating the application of a modified molecular beacon to search for potential inhibitors of BER.

We then tested whether, such oligonucleotide substrates with different DNA lesions are specific for particular DNA repair enzyme(s) in cell-free extracts. The mammalian alkyl-*N*-purine-DNA glycosylase (ANPG) excises a variety of damaged bases including alkylated purines and hypoxanthine (Hx) [2] and the DNA glycosylase activity towards a radioactively labelled Hx containing oligonucleotide was greatly decreased in mouse cells lacking ANPG [27,28]. Biochemical and genetic experiments show that the two AP endonucleases Apn1 and Apn2 from *S. cerevisiae* incise DNA containing an AP site analog, tetrahydrofuran (THF), and the *Δapn1 Δapn2* double mutant is deficient in the repair of AP sites [29,30]. To measure ANPG and Apn1/Apn2 activities, we designed FD-INO containing multiple hypoxanthine residues and FD-THF containing a

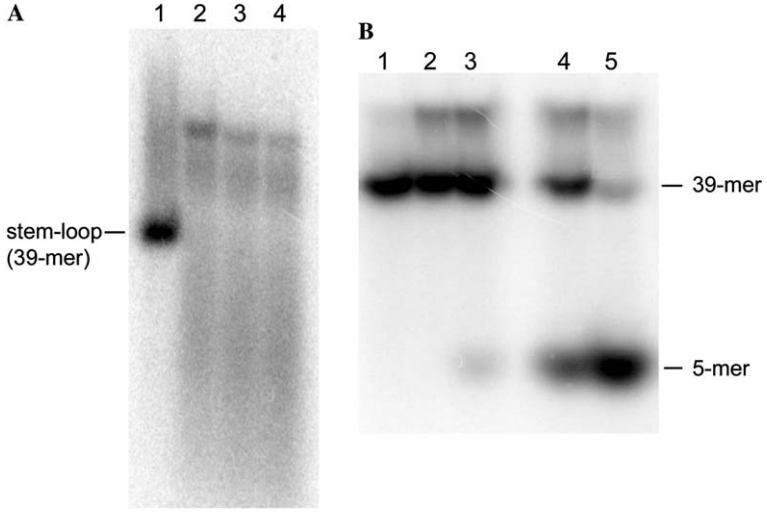

Fig. 1. Activity of hUNG (A) and APE1 (B) on 5′-^{32}P-labelled oligonucleotides. (A) 5′-^{32}P-labelled SL-UDG (10 nM) was incubated at 37 °C for 10 min with hUNG (1–100 nM). Lanes: 1, no enzyme; 2, 1 nM; 3, 10 nM; and 4, 100 nM. (B) 5′-^{32}P-labelled SL-APE1 (30 nM) was incubated at 37 °C for 5 min with APE1 (10 pM). Lanes: 1, no enzyme; 2, 1 nM; 3, 10 nM; and 4, 100 nM. For details see Materials and methods.

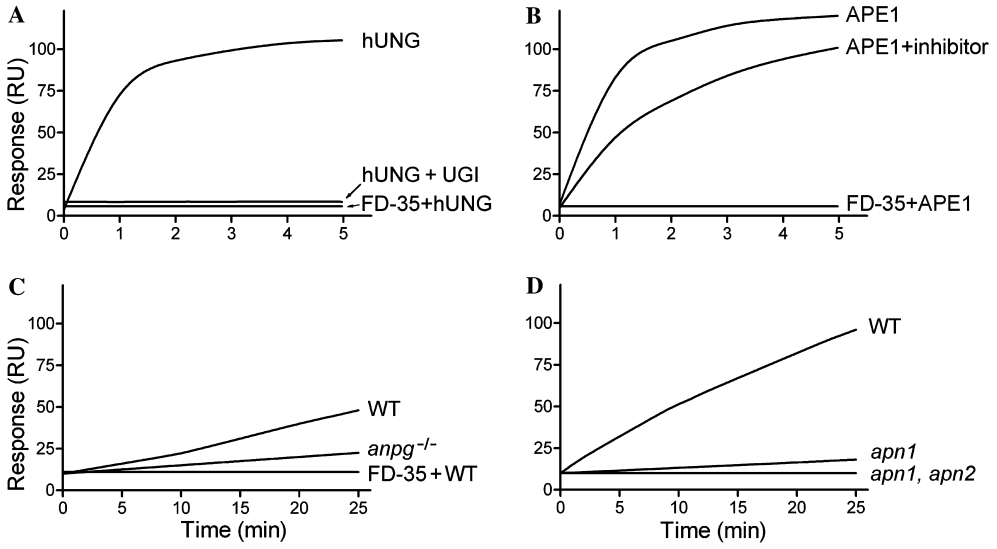

Fig. 2. Activity of hUNG, APE1, and cell-free extracts towards molecular beacon oligonucleotides containing modified bases. Fluorescence was expressed as response units (RU). FD-35, which contains normal bases, was used as a control. (A) Ten nanomolar FD-UDG was incubated at 37 °C for the time indicated with 1 nM hUNG with or without 0.1 U UGI. (B) Twenty nanomolar FD-APE1 (pre-treated with hUNG/$NaBH_4$) was incubated at 37 °C for the indicated time with 10 pM APE1, with or without 1 nM HDP. (C) Twenty nanomolar FD-INO was incubated at 22 °C with 200 μg of cell-free extracts from APNG+/+ or APNG−/− MEFs. (D) Twenty nanomolar FD-THF was incubated at 22 °C with 20 μg of cell-free extracts from wildtype (WT) or mutants of *S. cerevisiae*. For details see Materials and methods.

single THF residue at position 5, respectively. As shown in Fig. 2C, incubation of FD-INO, but not FD-35, with cell-free extracts from APNG+/+ MEFs reveals a time-dependent increase in fluorescence. As expected, the increase in fluorescence was strongly reduced (but not to background levels) when FD-INO was incubated with APNG−/− extracts. This observation suggests the presence of back-up repair activity(ies) towards Hx, not yet identified, in mammalian cells. The results in Fig. 2D show that the fluorescent signal generated by FD-THF has an absolute requirement for the *apn1* and *apn2* gene products consistent with previous data [29,30].

To further substantiate the specificity and efficiency of the beacon-based DNA repair assay, we compared the kinetic constants obtained on radioactively labelled oligonucleotides and modified beacons. FD-UDG and FD-THF were used as substrates for hUNG and APE1, respectively. Excision kinetics based on the beacon assay were shown to obey the Michaelis–Menten equation (data not shown). As shown in Table 1 the K_M, k_{cat}, and k_{cat}/K_M values for APE1 obtained using either beacon or ^{32}P-labelled oligonucleotides are very similar, whereas the K_M and k_{cat} constants for hUNG acting upon FD-UDG are much lower than those obtained on radioisotope-labelled oligonucleotides. This discrepancy may result from the difference in the number of uracil residues per molecule of substrate. More than one consecutive action of hUNG is needed to induce fluorescence on FD-UDG, whereas removal of a single uracil from ^{32}P-labelled oligonucleotides is sufficient for detection. Nevertheless, the efficiency (k_{cat}/K_M) ratios are very similar for hUNG on both substrates and taken together, these results suggest that the new method is sufficiently accurate and can be used for kinetic measurements.

Little is known about BER in living cells due to the lack of a suitable method for real-time imaging. To address this problem, FD-35, FD-UDG, FD-INO, and FD-THF beacons were transfected into HeLa and NIH3T3 cells and the change in fluorescence was monitored by fluorescence microscopy on fixed living cells. In addition, we used the fluorescent oligonucleotide (FL-35) without a quencher residue to control transfection efficiency and a beacon containing an 8-oxoguanine/adenine base pair (FD-8oxoG · A) to monitor mismatch-specific adenine-DNA glycosylase activity

Table 1
Kinetic constants of ANPG, hUNG, and Ape1 proteins on modified beacon (FD) and ^{32}P-labelled oligonucleotide duplexes

Enzyme	Substrate[a]	K_M (nM)	k_{cat} (min^{-1})	k_{cat}/K_M ($nM^{-1}\,min^{-1}$)
hUNG	FD-UDG	60 ± 12	510 ± 50	8.5
hUNG	[^{32}P]	1600 ± 0.7	$11{,}860 \pm 130$	7
Ape1	FD-Ape1	15 ± 2	908 ± 30	61
Ape1	[^{32}P]	20 ± 2	700 ± 20	35

[a] Kinetic constants for beacon and ^{32}P-labelled oligonucleotide were measured under the same conditions.

[31]. As shown in Fig. 3, only background levels of fluorescence were observed in control non-transfected HeLa cells (A) and in cells transfected with FD-35 (G). However, cells transfected with FL-35 (Fig. 3F), which lacks the 3′-quencher, exhibited high levels of fluorescence. These results indicate that beacon oligonucleotides can be efficiently delivered to mammalian cells, and that non-modified beacons are stable in vivo at least over the period of the experiment. High levels of fluorescence were also observed in the HeLa cells transfected with FD-UDG (Fig. 3B) and FD-THF (Fig. 3E), suggesting that the beacons are recognized and processed by uracil–DNA glycosylases and AP endonucleases in living cells with high efficiency. Conversely, lower levels of fluorescence were observed in cells transfected with FD-INO (Fig. 3C) and FD-8oxoGA (Fig. 3D), suggesting that human cells process the uracil residues and AP sites faster than Hx and mismatched adenine residues when present in DNA. Similar results were obtained when using mouse NIH3T3 cells: the cells transfected with FD-UDG, FD-INO, and FD-THF, but not with FD-35, showed a strong increase in fluorescence (Fig. 4). As shown in Fig. 5, in agreement with results obtained with living cells, the HeLa cell-free extract incises the AP sites faster than mismatched adenine residues.

Because of the artificial structure and the fluorophore-quencher pair the sensitivity of the molecular beacon assay may be different from that of the radioactively labelled bi-molecular DNA duplex. However, it should be noted that the thermodynamic stability of mono-molecular DNA duplex, in contrast to bi-molecular duplex, does not depend on DNA concentration [32]. Indeed, we were able to detect APE1 activity using only 0.1 nM of FD-THF, indicating that molecular beacons provide a highly sensitive assay (data not shown). Furthermore, several studies have shown that

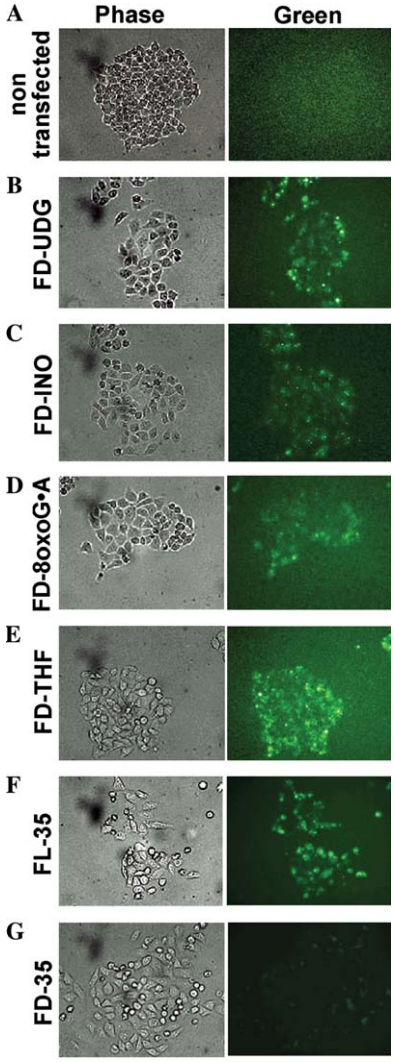

Fig. 3. Fluorescence emission of HeLa cells 5 h after transfection with various molecular beacon oligonucleotides (200 nM). Fixed and living HeLa cells were examined by phase and fluorescence microscopy. For details see Materials and methods.

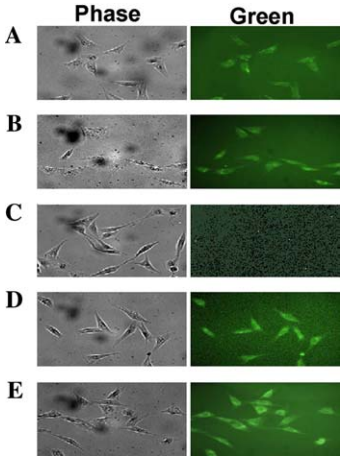

Fig. 4. Fluorescence emission of NIH 3T3 cells 5 h after transfection with various molecular beacon oligonucleotides (200 nM). Fixed and living NIH 3T3 cells were examined by phase and fluorescence microscopy. (A) FL-35, (B) FD-UDG, (C) FD-35, (D) FD-INO, and (E) FD-THF. For details see Materials and methods.

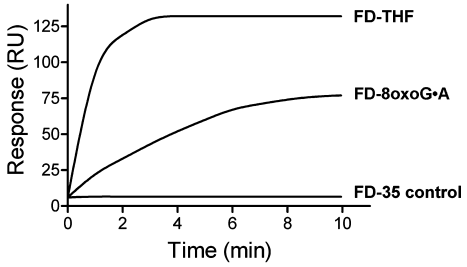

Fig. 5. AP-endonuclease and DNA glycosylase (MYH) activity of HeLa cell-free extracts (1 mg/mL) assayed using molecular beacon oligonucleotides FD-THF and FD-8oxoG · A, respectively. Molecular beacon oligonucleotide FD-35, which does not contain modified bases, was used as a control. For details see Materials and methods.

the minimal size of DNA duplex for efficient interaction with a DNA glycosylase and/or an AP endonuclease is between 11 and 16 bp in length [33–35]. Based on these observations and the presented data we propose that the modified molecular beacons with a short stem–loop region can interact in specific manner with base excision repair proteins.

In this initial report we have shown that molecular beacon oligonucleotides containing specific DNA damage are effective substrates for measuring DNA repair activities. Not only do they lend themselves to a high-throughput format for in vitro studies, but our preliminary data also indicate that they will be valuable tools for the determination of DNA repair functions in living cells. In this latter respect, molecular beacons should provide a relatively cheap and rapid, yet robust, approach to determe the existence of back-up repair activities in cells lacking specific DNA repair functions.

Acknowledgments

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