ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 2, pp. 245-252. © Pleiades Publishing, Ltd., 2011.
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Rapid Photometric Detection of Thymine Residues Partially Flipped out of Double Helix as a Method for Direct Scanning of Point Mutations and Apurinic DNA Sites

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Received June 26, 2010
Revision received September 19, 2010

Abstract—A spectroscopic assay for detection of extrahelical thymine residues in DNA heteroduplexes under their modification by potassium permanganate has been developed. The assay is based on increase in absorbance at 420 nm due to accumulation of thymidine oxidation intermediates and soluble manganese dioxide. The analysis was carried out using a set of 19-bp DNA duplexes containing unpaired thymidines opposite tetrahydrofuranyl derivatives mimicking a widespread DNA damage (apurinic (AP) sites) and a library of 50-bp DNA duplexes containing all types of base mismatches in different surroundings. The relation between the selectivity of unpaired T oxidation and the thermal stability of DNA double helix was investigated. The method described here was shown to discriminate between DNA duplexes with one or two AP sites and to reveal thymine-containing mismatches and all noncanonical base pairs in AT-surroundings. Comparative results of CCM analysis and the rapid photometric assay for mismatch detection are demonstrated for the first time in the same model system. The chemical reactivity of target thymines was shown to correlate with local disturbance of double helix at the mismatch site. As the spectroscopic assay does not require the DNA cleavage reaction and gel electrophoresis, it can be easily automated and used for primary screening of somatic mutations.

DOI: 10.1134/S0006297911020118

Key words: DNA structure, noncanonical pairs, heteroduplexes, chemical modification of heterocyclic bases, detection of mutations, apurinic sites

Genetic point mutations, being substitutions, insertions, or deletions of one or several base pairs, play a key role in the development of malignant and hereditary diseases. Single nucleotide substitutions associated with carcinogenesis may emerge at practically any site of the conservative region of the tumor growth suppressor gene, resulting in dysfunction of the gene product. Obviously, detection of point mutations that can be considered as molecular markers of tumor is of primary importance for tumor diagnostics and monitoring and for the choice of therapeutic strategy. Besides, detection of genomic mutations seems to be important for realizing personalized medicine, because mutations are often genetic variants associated with susceptibility to various diseases and drug response of an organism. For detection of mutations in already known positions, so-called "hot spots", a number of highly sensitive methods have been developed [1-4]. Regarding detection of point mutations of unknown localization, these approaches are usually supposed to be scanning techniques. At present, none of the numerous methods for revealing point mutations of unknown localization is fully satisfactory, i.e. they do not reveal all types of mutations and are not sensitive enough to "visualize" mutant DNA in the presence of normal DNA. So, direct sequencing [5, 6] reveals a mutation only if the amount of mutant DNA in the analyzed sample is no less than 30%, and the analysis of conformational polymorphism of single-stranded DNA [5, 7] is characterized by a high level of false-negative results as it reveals only mutations causing changes in the spatial conformation of a single-

Abbreviations: AP, apurinic site; CCM, Chemical Cleavage of Mismatches; TEAC, tetraethylammonium chloride; T_{ml}, melting temperature; prefix "d" in designation of sequences of oligodeoxyribonucleotides is omitted.

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stranded DNA fragment. Chemical Cleavage of Mismatches (CCM) is considered as one of the promising methods for detection of point mutations of unknown localization [8, 9]. It is based on formation of heteroduplexes of mutant and normal DNA fragments. Such heteroduplexes contain a noncanonical base pair at the point of mutation, which is revealed by chemical reactions sensitive to the structure of nucleic acids. The presence of an internal unpaired residue or noncanonical base pair weakening the stacking interactions results in extrahelical location of a heterocyclic base, making it a target for chemical reagents, the attack of which is perpendicular to the base plane. The subsequent cleavage of the polynucleotide chain at the modification site is revealed by electrophoresis in denaturing polyacrylamide gel. In spite of the clear theoretical basis, the CCM method is not extensively used for detection of nucleotide substitutions because of the large number of manipulations required and the need to use toxic chemical reagents. Besides, the absence of data on the reactivity of various noncanonical DNA pairs has prevented estimation of obtaining false-positive and false-negative results. Up to now the positive control has been a set of random samples with mismatches revealed by direct sequencing [10-12].

Versatile model systems based on synthetic oligonucleotides containing all types of noncanonical pairs in different microenvironments have been obtained in our previous work for estimation of reliability and sensitivity of the CCM method [13, 14]. Using the constructed system of positive controls, it has been shown that the reactivity of different noncanonical pairs unambiguously correlates with the degree of local perturbances that they introduce into the double-helical structure. The patterns of cleavage products in denaturing polyacrylamide gel are typical of each type of DNA mismatch in both AT- and GC-surroundings and can be checkpoints for the analysis of clinical samples. The Scion Image software for electrophoregram data processing was used to obtain quantitative data of the DNA chemical cleavage efficiency depending on the temperature and duration of treatment with the chemical reagent. These data were used to describe the modification kinetics of a number of mismatches, to optimize the conditions of CCM analysis, and to determine the limits of its sensitivity.

In addition to methods determining the type and position of point mutations, there is often a demand for methods of primary DNA screening to give a prompt answer to the question about the absence or presence of mutations. In this context, a promising approach is photometric detection of thymine-containing noncanonical pairs. This method proposed by Cotton's laboratory [15, 16] is based on the principles of CCM analysis but, contrary to the latter, does not require the stage of DNA cleavage by piperidine and electrophoretic separation of the resulting fragments. It is based on variation in the optical density of solutions as a result of interaction between extrahelical thymine residues and potassium permanganate. The absence of data on sensitivity and selectivity of rapid photometric detection of point mutations prevents full estimation of its advantages and disadvantages.

In the present work 19-bp DNA duplexes have been used for verification of the versatility of this method and for selection of its optimal conditions. These duplexes contained one or two unpaired thymidine residues opposite a tetrahydrofuranyl derivative in the complementary chain, which mimics a widespread DNA damage – the apurinic site. With short heteroduplexes it was possible to increase the share of thymine residues and to enhance the observed optical effect. Noncanonical pairs were analyzed photometrically with a collection of 50-bp DNA duplexes previously used for the study of reactivity of all mismatch types in different microenvironments by the CCM method. Within the framework of this study, the results of CCM analysis have been compared for the first time with the results of a rapid photometric method for mismatch scanning in the same model system.

MATERIALS AND METHODS

Oligodeoxyribonucleotides without modifications were obtained by solid-phase phosphoramidite synthesis (Syntol, Russia). Modified residues modeling AP sites were introduced into oligonucleotides by substituting 2-(dimethoxytrityloxymethyl)tetrahydrofuran-3-(*N*,*N*-diisopropylamide)-β-cyanoethylphosphite for the standard phosphoramidite. The original method of synthesis of such derivatives is described in [17]. Oligonucleotides containing a trityl group were isolated by reverse-phase HPLC [18]. They were finally purified by electrophoresis in denaturing polyacrylamide gel.

Heteroduplex formation. For obtaining 19-bp DNA duplexes du-1AP and du-2AP containing one or two apurinic sites opposite thymidine residues, respectively (Table 1), the oligonucleotide components were mixed in an equimolar ratio in buffer A (10 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM NaCl, pH 7.6). The oligonucleotides were hybridized for 2-3 h by slow cooling from 80 to 5°C. The unmodified control duplex du-K with adenosine residues instead of the AP sites was obtained in a similar way.

The sets of homo- and heteroduplexes with all variants of mismatches including single-nucleotide deletions (internal unpaired bases) were obtained by combining oligonucleotides of direct and reverse sequences in an equimolar ratio up to the final concentration of 10 ng/μl in buffer B (1 M NaCl, 2 mM EDTA, 6 mM Tris-HCl, pH 7.5). Oligonucleotides were hybridized by the following program: at 95°C for 2 min; 25 cycles of temperature decrease by 2°C every 30 sec (at 95°C for 30 sec; at 93°C for 30 sec, etc.); at 42°C for 1 h; temperature decrease to 25°C.

Table 1. Melting parameters of DNA duplexes containing AP sites

DNA duplex*	Designation	Character of helix–coil transition	T_{ml}** ± 1°C
5' GGAAAATTTCAGCAAGGTG 3' CCTTTTAAAGTCGTTCCAC	du-K	one-step	64
5' GGAAAATTTC**Y**GCAAGGTG 3' CCTTTTAAAGTCGTTCCAC	du-1AP	two-step	41, 55
5' CGAAAATTTC**Y**GC**Y**AGGTG 3' CCTTTTAAAGTCGTTCCAC	du-2AP	one-step	45

* **Y**, tetrahydrofuran analog of apurinic site: 2-(hydroxymethyl)tetrahydrofuran-3-ol. Duplex structure formed due to fully complementary 10-bp regions of the direct and reverse chains is underlined.

** The table gives temperatures of helix–coil half transitions.

The oligonucleotides for obtaining homo-/heteroduplexes with the mismatch surrounded by A·T pairs were as follows:

direct sequence – (5′-3′) CGGCGGCCTTGTGGTAGTTGGACA**TXT**AGGCGTAGGCAAGAGTCGCCCGG, where X is G (1), C (2), A (3), T (4), deletion (5); numbers of oligonucleotides involved in pair-wise hybridization are given in parentheses;

reverse sequence – (5′-3′) CCGGGCGACTCTTGCCTACGCCTA**Y**ATGTCCAACTACCACAAGGCCGCCG, where Y is C (6), G (7), A (8), T (9), deletion (10).

The oligonucleotides for obtaining homo-/heteroduplexes with mismatch surrounded by G·C pairs were as follows:

direct sequence – (5′-3′) CGGCGGCCTTGTGGTAGTTGGACA**GXG**AGGCGTAGGCAAGAGTCGCCCGG, where X is G (11), C (12), A (13), T (14), deletion (15);

reverse sequence – (5′-3′) CCGGGCGACTCTTGCCTACGCCT**CYC**TGTCCAACTACCACAAGGCCGCCG, where Y is C (16), G (17), A (18), T (19), deletion (20).

Variants of nucleotide mismatches obtained by pairwise hybridization of oligonucleotides under the respective numbers are given in Table 2.

Spectrophotometric detection of apurinic sites in 19-bp DNA duplexes. Kinetic curves were obtained as follows: 200 μl of 1.5 mM $KMnO_4$ solution in 4.5 M TEAC was added to 100 μl of solution of DNA homo- or heteroduplex in buffer A in a 1-cm quartz cuvette and quickly stirred. We used micro, self-masking cuvette so that the final sample volume (300 μl) was enough for spectrophotometric measurement. The kinetics of optical density changes at 420 nm was registered using a Cary 500 Bio UV-VIS spectrophotometer (USA). Spectra in the region of 440-410 nm were recorded with an interval of 0.5 min at 15°C. After 1 h, the reaction was stopped by adding 20 μl of solution (0.5 ml of this solution contained 70 μl EtSH, 25 μl tRNA (1 mg/ml), and 250 μl 3 M NaOAc); the nucleotide material was precipitated with ethanol. The experiment was repeated three times to determine reproducibility of the results. As a preliminary, the optimal DNA duplex concentration was determined: 20 pmol/100 μl. Before the reaction, the actual DNA concentration in the sample was measured (by spectrophotometry in the UV region) and optical density values at 420 nm were normalized when plotting the kinetic curves, i.e. brought to the concentration 100 pmol/ml.

Heteroduplexes du-1AP and du-2AP were cleaved at the modified regions after their treatment with 1.5 mM $KMnO_4$ solution in 4.5 M TEAC, spectrophotometry, and separation from the reaction mixture by precipitation with 96% ethanol. The DNA was reprecipitated and treated with 10% piperidine at 90°C for 20 min. The piperidine was evaporated in a SpeedVac Concentrator (Savant). For complete removal of piperidine, the procedure was repeated twice with dissolving of the sample in 20 μl of 50% ethanol. Heteroduplex cleavage products were separated by electrophoresis in 20% polyacrylamide gel containing 7 M urea for 50 min at 800 V. The sample was pre-dissolved in 5 μl of 80% formamide solution containing 10 mM EDTA and the marker dyes. The bands corresponding to oligonucleotides were revealed by staining the gel with a SYBR Gold fluorescent dye (Invitrogen, USA) for 30 min in 100 μl of TE buffer (100 mM Tris-HCl, 10 mM EDTA) containing 1 μl of the dye stock solution. The gel was scanned using a Fujifilm Fla-3000 scanner (USA) at the excitation wavelength of 450 nm. The fluorescence emission wavelength was 537 nm.

Noncanonical pairs and unpaired bases in 50-bp DNA duplexes were detected spectrophotometrically just as for revealing AP sites in the shorter DNA duplexes. However, in this case the concentration of nucleotide material was increased to 100 pmol of the duplex in 100 μl of buffer B and spectra were measured at 25°C. When plotting the

Table 2. Variable central region of 50-mer oligodeoxyribonucleotides used for obtaining all types of homo- and heteroduplexes by pair-wise hybridization with a complementary partner

Oligonucleotide		Direct sequence				
		1 -A**T**G**T**A-	2 -A**T**C**T**A-	3 -A**T**A**T**A-	4 -A**T**T**T**A-	5 -A**T**-**T**A-
Reverse sequence	6-T**A**C**A**T-	1 G·C*	6 C·C	11 A·C	16 T·C	21 _C
	7-T**A**G**A**T-	2 G·G	7 C·G	12 A·G	17 T·G	22 _G
	8-T**A**A**A**T-	3 G·A	8 C·A	13 A·A	18 T·A	23 _A
	9-T**A**T**A**T-	4 G·T	9 C·T	14 A·T	19 T·T	24 _T
	10-T**A**-**A**T-	5 G _	10 C_	15 A _	20 T_	25 _/_-
Oligonucleotide		Direct sequence				
		11 -A**G**G**G**A-	12 -A**G**C**G**A-	13 -A**G**A**G**A-	14 -A**G**T**G**A-	15 -A**G**-**G**A-
Reverse sequence	16-T**C**C**C**T-	26 G·C	31 C·C	36 A·C	41 T·C	46 _C
	17-T**C**G**C**T-	27 G·G	32 C·G	37 A·G	42 T·G	47 _G
	18 -T**C**A**C**T-	28 G·A	33 C·A	38 A·A	43 T·A	48 _ A
	19 -T**C**T**C**T-	29 G·T	34 C·T	39 A·T	44 T·T	49 _T
	20 -T**C**-**C**T-	30 G_	35 C_	40 A _	45 T_	50 _ /_

* Numbers of DNA duplexes and type of variable pair or unpaired base.

kinetic curves all data were normalized and brought to the concentration 100 pmol/ml.

RESULTS

The advantage of photometric detection of extrahelical thymine residues is as follows: the analysis requires neither the cleavage of the DNA strand nor electrophoretic separation of fragments, and the only reagent is the readily available and nontoxic potassium permanganate. The disadvantages of this method are the impossibility of determining the mutation site and some limitations in versatility due to the fact that only thymine residues flipped out of the double-helical structure undergo chemical modification. Using the complete set of 50-bp DNA duplexes with all types of noncanonical base pairs and bulge bases, we have shown that potassium permanganate allows identification of not only T-containing mismatches but also any noncanonical pairs in AT-surroundings [13, 14]. This phenomenon is due to disturbance in the local structure of the double helix affecting the adjacent bases that is induced by the noncanonical pair. In doing so the targets of $KMnO_4$/TEAC are thymine bases of not only a defective pair, but also of the immediate surroundings. If the mismatch is flanked by G·C pairs, potassium permanganate can reveal only thymine-containing mismatches [13, 14].

Photometric detection of AP sites. Since the internal unpaired residue T is a perfect target for $KMnO_4$, versatility of the photometric method was verified and its optimal conditions were selected using 19-bp DNA duplexes containing one or two thymine residues opposite a tetrahydrofuranyl derivative in the complementary chain mimicking a widespread type of DNA damage: the apurinic site (Table 1). In contrast to 2-deoxyribofuranose, isosteric tetrahydrofuran is resistant to β-elimination. Short heteroduplexes make it possible to increase the share of thymine residues sensitive to potassium permanganate attack and to enhance the observed optical effect. DNA duplexes were treated directly in the cuvette, and the time-dependent registration of optical density of the solution made it possible to obtain the kinetic curves of oxidation of "flipped out" thymine residues in different systems (Fig. 1). One can see that in 30 min at 15°C the absorbance (420 nm) in the reaction mixture with heteroduplex du-1AP (Table 1) containing a single AP site exceeds 2.2-fold the absorbance of the homoduplex du-K sample. For the DNA duplex with two AP sites, the discrimination factor increased to 4.6. The kinetic curves for both modified duplexes practically plateaued. Absorption of a DNA-free sample was insignificant. Consequently,

reduction of potassium permanganate in air had no effect on the results.

The reaction temperature was of particular significance for this system. It is impossible to obtain the correct kinetic curves for potassium permanganate oxidation of a heteroduplex with two unpaired thymines at 25°C because all thymine residues are completely modified under these experimental conditions. It is known that the presence of an apurinic site disturbing the stacking interaction between heterocyclic bases strongly destabilizes the double helix [19]. The study of thermal stability of DNA duplexes du-1AP, du-2AP, and du-K in work [20] showed that the shape of melting curves (one- or two-step), the melting temperature, and the cooperativity of helix–coil transition substantially depend on the number, position, and microenvironment of apurinic sites. So, a region containing two apurinic sites is already melted at room temperature, while duplex structure is formed due to fully complementary 10-bp regions of the direct and reverse chains (underlined in Table 1). The shape of the kinetic curve of oxidation of duplex du-2AP at 25°C (data not shown) suggests that $KMnO_4$ reacts not only with T residues located opposite the two apurinic sites, but also with the thymines of single-stranded ends of the DNA duplex. Oxidized T residues additionally loosen the structure, which gradually opens, making all thymine bases of both strands accessible for attack. This conclusion was confirmed by electrophoretic analysis of DNA fragments obtained as a result of reaction between du-2AP and potassium permanganate at 25°C followed by treatment with piperidine. However, only partial cleavage of the heteroduplex with two AP sites at thymine residues was observed at 15°C, yielding a typical ladder (Fig. 2, lane *4*). It should be noted that DNA was detected with SYBR Gold fluorescent dye visualizing all cleavage fragments of both chains. The fact that 10-mer oligonucleotides were registered in the system containing the heteroduplex with a single AP site (Fig. 2, lane *3*) substantiates the specificity of modification of unpaired thymine bases under conditions of double helix stability.

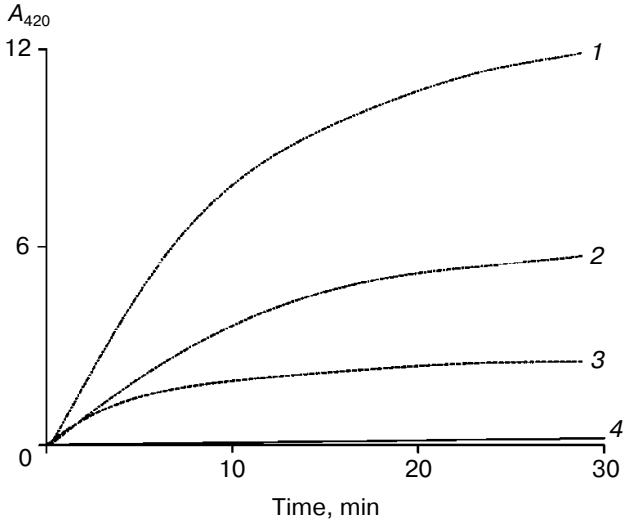

Fig. 1. Kinetics of photometric scanning at 420 nm of DNA duplexes du-2AP (*1*), du-1AP (*2*), and du-K (*3*) (Table 1) after treatment with potassium permanganate at 15°C. Curve *4* corresponds to a DNA-free sample. Optical density values are normalized to the concentration of oligonucleotide duplexes 100 pmol/ml. Reaction conditions are described in "Materials and Methods".

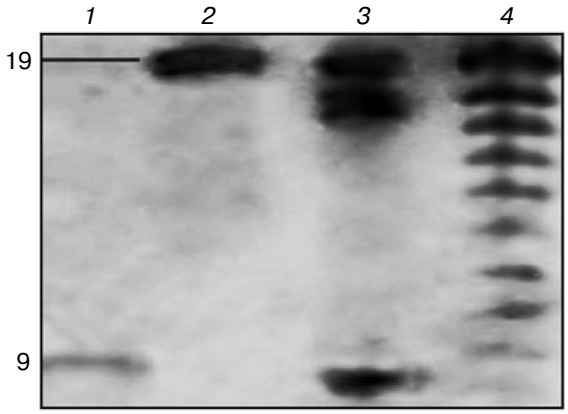

Fig. 2. Electrophoretic separation of the products of piperidine cleavage of DNA duplexes du-K (lane *2*), du-1AP (lane *3*), and du-2AP (lane *4*) (Table 1) after 30-min treatment with potassium permanganate at 15°C. The 9-mer oligonucleotide (lane *1*) was used as a control. Oligomers were separated in denaturing polyacrylamide gel; DNA was detected by staining with SYBR Gold fluorescent dye allowing visualization of all oligonucleotide fragments. The length of oligodeoxyribonucleotides is shown on the left.

Thus, it has been shown that the photometric analysis not only ascertains the presence of unpaired thymine but also discriminates DNA duplexes with one or two apurinic sites.

Detection of noncanonical pairs and internal unpaired bases by the photometric method. The stability of short duplexes substantially influences the results of their study. This difficulty disappears during the analysis of longer double helices. We used a set of 50-bp DNA duplexes containing all types of noncanonical pairs and internal unpaired bases in the AT- or GC-surroundings (Table 2). The optimal conditions for potassium permanganate oxidation of homo- and heteroduplexes of this series were selected to enable detection of not only unpaired T residues, but also different noncanonical pairs disturbing the double helix structure to a lesser extent.

It was shown that the 50-bp DNA duplexes containing all types of base mismatches in the same position are modified much slower than the 19-bp duplexes; therefore, experimental DNA concentration was increased to 100 pmol in 100 μl of solution. The kinetic curves for the series of DNA duplexes, where the variable pair (unpaired base) was present in the AT-environment, obtained by photometric detection, are presented in Fig. 3 (see color

inset). One can see that the absorption of homoduplex solution at 420 nm is much less than the absorption observed for heteroduplex solutions and, within the experimental error, the kinetic curves of homoduplexes differing in a single central nucleotide pair (A·T (duplex 14), T·A (duplex 18), G·C (duplex 1), and C·G (duplex 7)) coincide. However, heteroduplexes of this series show a very large difference in the run of their kinetic curves. The most intensive absorbance in 30 min of the reaction with potassium permanganate, i.e. the most efficient oxidation of thymine residues, is observed for the heteroduplex with T·T mismatch; it is followed by DNA duplexes with internal unpaired thymines: T_ and _T. Then, in the order of light absorption decrease, there are heteroduplexes with the following noncanonical pairs and unpaired bases: _C > T·C > _G > C_ > A_ = C·T > G_ > _A > A·A > C·C > A·C > C·A > T·G > G·A = G·G = A·G > G·T.

These results correlate with the data obtained by the method of chemical modification and mismatch cleavage in the same model system. The degree of the cleavage of DNA heteroduplex chains with thymine-containing noncanonical pairs and unpaired thymine residues decreases in the following order: T_ > _T > T·T > T·C > C·T > T·G > G·T [14]. The data of CCM analysis do not include heteroduplexes where mismatched bases are represented by residues A, C, and G surrounded by A·T pairs because in these cases it is difficult to obtain exact quantitative characteristics of the DNA cleavage degree. As opposed to the above, the kinetic curves obtained in this work give a visual image of base modification and provide for easy comparison of different mismatch-containing duplexes with each other. It can be seen that the order, where the intensity of chemical DNA cleavage drops, practically coincides with the trend revealed by photometric scanning of noncanonical pairs. In both cases, the minimum intensity of modification (proportional either to absorbance or to the degree of DNA cleavage) was observed for the noncanonical Wobble-pair G·T. The highest absorption or, respectively, cleavage was shown for the unpaired residue T and thymine as components of T·T and T·C mismatches. The duplexes with mismatches not containing thymines but surrounded by A·T pairs are generally less liable to modification, because in this case the targets of potassium permanganate attack are not bases of the mismatched pair but thymine residues of the neighboring Watson–Crick base pairs. This was confirmed by analysis of the products of chemical DNA cleavage at the modified base pairs [13, 14]. It should be noted that the ability of thymine residues flanking DNA mismatch to react with potassium permanganate is of great practical use because it allows the detection of mismatched A, G, or C residues (which do not show sufficient reactivity towards $KMnO_4$) and, consequently, demonstrate the presence of genomic mutations. The kinetic curves of absorbance in the solution of DNA duplexes containing mismatches in a GC-environment are not presented; however, in this case photometric data also correlate with the results of CCM analysis and provide for unambiguous estimation of the presence/absence of a thymine-containing noncanonical base pair. Like in the experiments on chemical modification and cleavage of DNA, different intensities of photometric signals of the solutions of heteroduplexes with different types of mismatches correspond to the degree of structural disturbance introduced by various noncanonical pairs or unpaired bases into local structure of the double helix [14].

DISCUSSION

This work presents model systems on the basis of synthetic oligonucleotides for the analysis of reliability and sensitivity of a photometric method for detection of noncanonical pairs associated with point DNA mutations and unpaired thymine residues in apurinic sites (which are common DNA damages). It is based on analysis of the products of oxidation of partially flipped out thymine residues by potassium permanganate. It has been established that the targets for this reagent are thymines of apurinic sites, thymines of noncanonical pairs and unpaired bases, and thymines of mismatch-flanking Watson–Crick base pairs. The presence of T residues in the zone of structural disturbance, which are characterized by enhanced reactivity compared to the bases of canonical pairs, are detected by means of mere photometry in the course of oxidative modification of a sample by potassium permanganate. Oxidation of pyrimidine residue at the C5–C6 bond results in formation of intermediate hypomanganate diester and soluble manganese dioxide [21]. Formation of a pentameric cyclic activated complex is the rate-limiting stage and the main source of absorbance in the visible region at 420 nm [22, 23]. This wavelength was used for quantitative measurement of oxidation level and reaction rate [22, 24].

Selective recognition of T-containing mismatches is of great significance in the context of detection of point nucleotide substitutions, because thymine is involved in 49% of point mutations of the human genome and C–T transitions make up about 33% of all genomic substitutions [25].

As regards AP sites, these biologically active DNA damages appear as a result of spontaneous hydrolysis of *N*-glycoside bonds [26, 27] or removal of modified bases by DNA glycosylases [26, 28]. These damages stimulated the reaction of β-elimination resulting in uncontrolled cleavage of the polynucleotide chain [28-30]. It is established that AP sites are the main intermediates in chemical mutagenesis and in the process of DNA repair [26, 31, 32].

Chemical reagents that can selectively label the flipped out bases may be reliable tools for detection of thermodynamically weakened regions of the double helix

and for investigation of their role in various biological processes. In particular, $KMnO_4$ was used for the study of structural changes of DNA caused by different molecules (enzymes, hormones, receptors, intercalators, and drugs) and for identification of some noncanonical DNA forms (Z-DNA, double helix curvatures, hairpin structures, parallel-stranded DNA) [33]. Site-selective oxidation of bases, mainly thymine, in the specific region of DNA sequence underlies these approaches.

Although the principle of photometric scanning of flipped out T has been described, the present work is an example of complex systematic investigation of potential of the method based on a system of positive controls containing all types of noncanonical pairs and "excess" bases in the environment of the A·T or G·C pairs and the apurinic sites flanked by G·C pairs.

It is known that structural disturbances of the double helix generated by the AP site depend on the nucleotide context and nature of the base located opposite this site in the double helix. Experimental data and calculations of molecular dynamics demonstrate that disturbance of local DNA conformation is more marked in the presence of pyrimidine bases opposite the apurinic site [34]. In this work, the apurinic sites were analyzed in the series of 19-bp DNA duplexes containing one or two tetrahydrofuranyl residues mimicking the AP site opposite the unpaired thymine in the complementary chain. The AP sites were flanked with the G·C pairs not to create additional targets for $KMnO_4$ attack, which could occur in the case of neighboring A·T pairs. A relationship between selectivity of the oxidation of unpaired thymines and thermal stability of the double helix has been revealed. The conditions (DNA concentration, temperature mode) adapted to the analysis of short DNA were selected allowing distinct discrimination of DNA duplexes with one and two AP sites. The study has shown, however, that stability of short duplexes has a strong influence on the result.

In case of much longer 50-bp DNA duplexes, which we have used for the photometric analysis of noncanonical pairs, the limitation associated with different thermal stability of perfect and defective double helices is eliminated. Our preliminary studies of this system by the CCM method revealed the targets for $KMnO_4$ attack for all types of noncanonical pairs in AT- or GC-surroundings and enabled the correct interpretation of the photometric data. It should be noted that DNA concentration and the time of incubation with the reagent have to be substantially increased as the duplex length increases. For statistically significant results, DNA concentration was raised to 100 pmol in 100 μl. Analysis of obtained kinetic curves (Fig. 3) has shown that the optical absorption of heteroduplexes is 2-5 times higher than the absorption of the respective homoduplexes used as a control. Since not only thymines of noncanonical base pairs but also T residues within neighboring A·T pairs undergo modification, the kinetic curves reveal the integral effect of oxidation of all partially flipped out thymine bases, immediately providing data suitable for comparison. The correctness of the results of this research is determined by comparison of the sets of DNA duplexes with a single nucleotide substitution in the same position. The data of comparative experiments demonstrate that the photometric method quickly reveals the T-containing mismatches and mismatches involving other bases but flanked with A·T pairs. The discrimination factor of noncanonical base pairs and bulge bases at the level of 50-bp DNA duplexes varies from 5.1 for T·T mismatch to 1.9 for the G·T pair. Analysis of the literature [35-37] and our own results [13, 14] shows that the intensity of optical absorption of heteroduplex solution on addition of $KMnO_4$ correlates with the efficiency of chemical cleavage of the mismatch and corresponds to the degree of structural perturbation induced by various noncanonical pair or unpaired base. Kinetic curves were obtained that provide for correct comparison of the degree of local disturbances in the secondary structure caused by DNA mismatches or bulge bases.

Being an effective method of the primary screening of mutations of unknown localization, spectrophotometric detection, however, gives no information about the location of a nucleotide substitution, giving way to CCM analysis. Thus, the two methods supplement each other and can be used further in clinical practice.

This work was supported by the Russian Foundation for Basic Research (project No. 09-04-01315) and a grant for Leading Scientific Schools (3314.2010.4).

REFERENCES

1. Nakatani, K. (2004) *Chem. Bio. Chem.*, **5**, 1623-1633.
2. Latorra, D., Campbell, K., Wolter, A., and Hurley, J. M. (2003) *Human Mutation*, **22**, 79-85.
3. Brenner, E. V., Ivanova, E. M., Pyshny, D. V., and Morozov, I. V. (2005) *Bioorg. Khim.*, **31**, 213-215.
4. Karaman, M. W., Groshen, S., Lee, Ch.-Ch., Pike, B. L., and Hacia, J. G. (2005) *Nucleic Acids Res.*, **33**, e33.
5. Fan, X., Furnari, F. B., Cavenee, W. K., and Castresana, J. S. (2001) *Int. J. Oncol.*, **18**, 1023-1026.
6. Chan, E. Y. (2005) *Mutat. Res.*, **573**, 13-40.
7. Hongyo, T., Buzard, G. S., Calvert, R. J., and Meghorst, C. M. (1993) *Nucleic Acids Res.*, **21**, 3637-3642.
8. Cotton, R. G., Rodrigues, N. R., and Campbell, R. D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4397-4401.
9. Lambrinakos, A., Yakubovskaya, M., Babon, J. J., Neschastnova, A. A., Vishnevskaya, Y. V., Belitsky, G. A., D'Cunha, G., Horaitis, O., and Cotton, R. G. H. (2004) *Human Mutation*, **23**, 186-192.
10. Cotton, R. G. H., and Campbell, R. D. (1989) *Nucleic Acids Res.*, **17**, 4223-4233.
11. Roberts, E., Deeble, V. J., Woods, C. G., and Taylor, G. R. (1997) *Nucleic Acids Res.*, **25**, 3377-3378.

12. Tessitore, A., Di Rocco, Z. C., Cannita, K., Ricevuto, E., Toniato, E., Tosi, M., Ficorella, C., Frati, L., Gulino, A., Marchetti, P., and Martinotti, S. (2002) *Genes, Chromosomes & Cancer*, **35**, 86-91.
13. Neschastnova, A. A., Gasanova, V. K., Belitsky, G. A., Yakubovskaya, M. G., and Dolinnaya, N. G. (2007) *Mol. Biol. (Moscow)*, **41**, 535-543.
14. Yakubovskaya, M. G., Belyakova, A. A., Gasanova, V. K., Belitsky, G. A., and Dolinnaya, N. G. (2010) *Biochimie*, **92**, 762-771.
15. Bui, C. T., Lambrinakos, A., and Cotton, R. G. H. (2003) *Biopolymers*, **70**, 628-636.
16. Tabone, T., Sallmann, G., Webb, E., and Cotton, R. G. H. (2006) *Nucleic Acids Res.*, **34**, e45.
17. Volkov, E. M., Kubareva, E. A., Sergeev, V. N., and Oretskaya, T. S. (1990) *Khim. Prirod. Soedin.*, **3**, 417-419.
18. Dolinnaya, N. G., Jan, M. R., Kawde, A.-N., Oretskaya, T. S., Tashlitsky, V. N., and Wang, J. (2006) *Electroanalysis*, **18**, 399-404.
19. Gelfand, C. A., Plum, G. E., Grollman, A. P., Johnson, F., and Breslauer, K. J. (1998) *Biochemistry*, **37**, 7321-7327.
20. Hianik, T., Wang, X., Andreev, S., Dolinnaya, N., Oretskaya, T., and Thompson, M. (2006) *Analyst*, **131**, 1161-1166.
21. Simandi, L. I., and Jaky, M. (1976) *J. Am. Chem. Soc.*, **98**, 1995-1997.
22. Hayatsu, H., and Iida, S. (1969) *Tetrahedron Lett.*, 1031-1034.
23. Bui, C. T., Sam, L. A., and Cotton, R. G. (2004) *Bioorg. Med. Chem. Lett.*, **14**, 1313-1315.
24. Bui, C. T., and Cotton, R. G. H. (2002) *Bioorg. Chem.*, **30**, 133-137.
25. Zhang, F., and Zhao, Z. (2004) *Genomics*, **84**, 785-795.
26. Loeb, L. A., and Preston, B. D. (1986) *Ann. Rev. Genet.*, **20**, 201-230.
27. Lindahl, T. (1982) *Annu. Rev. Biochem.*, **51**, 61-87.
28. Weiss, B., and Grossman, L. (1987) *Adv. Enzymol. Relat. Areas Mol. Biol.*, **60**, 1-34.
29. Wu, X., and Wang, Z. (1999) *Nucleic Acids Res.*, **27**, 956-962.
30. Sudyina, A. E., Volkov, E. M., Oretskaya, T. S., Degtyarev, S. Kh., Gonchar, D. A., and Kubareva, E. A. (2000) *Bioorg. Khim.*, **26**, 442-447.
31. Demole, B., and Harrison, L. (1994) *Annu. Rev. Biochem.*, **63**, 915-948.
32. Cappelli, E., Degan, P., and Frosina, G. (2000) *Carcinogenesis*, **21**, 1135-1141.
33. Bui, C. T., Rees, K., and Cotton, R. G. H. (2003) *Nucleosides, Nucleotides & Nucleic Acids*, **22**, 1835-1855.
34. Chen, J., Dupradeau, F.-Y., Case, D. A., Turner, C. J., and Stubbe, J. (2008) *Nucleic Acids Res.*, **36**, 253-262.
35. Peyret, N., Seneviratne, P. A., Allawi, H. T., and SantaLucia, J., Jr. (1999) *Biochemistry*, **38**, 3468-3477.
36. Tanaka, F., Kameda, A., Yamamoto, M., and Ohuchi, A. (2004) *Biochemistry*, **43**, 7143-7150.
37. Tikhomirova, A., Beletskaya, I. V., and Chalikian, T. V. (2006) *Biochemistry*, **45**, 10563-10571.