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PCR synthesis of double stranded DNA labeled with 5-bromouridine. A step towards finding a bromonucleoside for clinical trials

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ABSTRACT

Incorporation of 5-bromouridine (5BrdU) into DNA makes it sensitive to UV and ionizing radiation, which opens up a prospective route for the clinical usage of 5-bromouridine and other halonucleosides. In the present work the polymerase chain reaction (PCR) protocol, which enables a long DNA fragment (resembling DNA synthesized in the cell in the presence of halonucleosides) to be completely substituted with 5BrdU, was optimized. Using HPLC coupled to enzymatic digestion, it was demonstrated that the actual amounts of native nucleosides and 5BrdU correspond very well to those calculated from the sequence of PCR products. The synthesized DNA is photosensitive to photons of 300 nm. HPLC analysis demonstrated that the photolysis of labeled PCR products leads to a significant decrease in the 5BrdU signal and the simultaneous occurrence of a uridine peak. Agarose and polyacrylamide gel electrophoresis suggest that single strand breaks and cross-links are formed as a result of UV irradiation. The PCR protocol described in the current paper may be employed for labeling DNA not only with BrdU but also with other halonucleosides.

1. Introduction

The enhanced sensitivity of DNA molecules labeled with 5-bromouridine (5BrdU) to ionizing radiation has been known for the last 40 years [1,2]. 5BrdU radiosensitizes genomic DNA to single and double strand breaks [3] and also to inter cross-links [4], the impairments that lead to chromosomal aberrations [5], a decreased rate of DNA repair processes of lethal damage [6] and eventually to cell death [7]. In several clinical trials, the enhanced response of non-hypotoxic tumor cells to radiation in the presence of bromodeoxyuridine has been recognized [8]. Some clinical studies have also reported radiosensitization of malignant brain tumors by 5BrdU [9]. Moreover, 5-fluorouracyl has been tested very recently for adjuvant chemoradiation therapy of gastric cancer in a series of very promising clinical studies [10].

The generally accepted mechanism of action of the halogen derivatives of pyrimidines (Hal-Pys) incorporated in DNA assumes the formation of the reactive pyrimidine-5-yl radical either due to the attachment of an excess electron [11] or the absorption of a photon [12]. Model studies on short DNA fragments comprising 5BrdU demonstrate the formation of single and double strand breaks as well as cross-links resulting from radiolysis in water solutions [4]. Similarly, photolysis of Hal-Pys labeled single and double stranded DNA with photons from 270 to 310 nm range indicates comparable damage to that identified within the above-mentioned radiolytic studies, i.e. single strand breaks and intra cross-links [11,12].

Despite the fact that the radio- and photosensitizing properties of Hal-NBs seem to be quite advantageous, the compounds have not yet been used in clinical practice [4]. It is probably not our fully understanding the details of the molecular mechanisms leading to DNA damage that prevents their rational use. Thus, before these substances can be incorporated into clinical practice, intensive studies are necessary to enable the sequence of events that leads to the labeled DNA damage induced by an excess electron or UV light to be elucidated. It is worth emphasizing that most model investigations carried out to date have employed only short DNA fragments containing 1–2 Hal-NBs per oligonucleotide molecule [4,11,12]. However, one should notice that DNA synthesized by a cell in the presence of Hal-NBs is substituted with halonucleobases to quite a large extent. For instance, it has been demonstrated that almost every fifth thymidine was substituted with 5-iodouracil in the granulocytes of patients suffering from high-grade gliomas, sarcomas, or locally advanced non-central nervous system tumors

Abbreviations: 5BrdU, 5-bromouridine; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography; Hal-Pys, halogen derivatives of pyrimidines; Hal-NBs, halogenated nukleobases; Taq, polymerase from *Thermus aquaticus*; Pfu, polymerase from *Pyrococcus furiosus*; Pwo, polymerase from *Pyrococcus woesei*; DNase I, deoxyribonuclease I; SVP, snake venom phosphodiesterase; BAP, bacterial alkaline phosphatase; DMSO, dimethyl sulfoxide.

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[13]. Moreover, the length of oligonucleotides obtained by chemical synthesis is limited to ca. 200 monomers [14,15], which is significantly shorter than the length of genomic DNA. Additionally, short synthetic DNA fragments seem to be quite a crude model of natural DNA, since the effects connected with the folding of natural DNA fragments (e.g. supercoiled DNA) could have a profound influence on the susceptibility of the chemical bonds in DNA to damage. Yet another difficulty concerning the usage of chemically synthesized oligonucleotides is related to the fact that such material is contaminated with DNA fragments of different length as well as with chemical impurities. Finally, the cost of chemical substitution is quite high and rises quickly with the number of substituted nucleotides. Therefore, the lack of a relatively cheap and convenient source of a well-defined material resembling *in vivo* labeled DNA seems to be a factor discouraging model studies that could lead to conclusions relevant for the cell.

The above facts prompted us to work out a modified PCR protocol enabling all thymidines (except those from primers) to be substituted with 5BrdU in a relatively long (500 bp) DNA fragment. The polymerase chain reaction (PCR) is one of the most widely used techniques of molecular biology for simply and rapidly producing microgram amounts of DNA, and the chosen PCR methodology is free of the mentioned above limitations. Indeed, "long and accurate" (LA) PCR enables the cost-effective synthesis of even 31 kb fragments from human genomic DNA and up to 42 kb fragments from bacteriophage lambda DNA [16,17]. Because of the high substrate specificity of DNA polymerase, only a limited number of modified nucleotides have been reported as a substrate for the modified DNA synthesis by PCR [18–20]. There are at least two factors that affect the PCR amplification of modified DNA: (i) a modified nucleoside triphosphate, which should be a good substrate for thermostable DNA polymerase, and (ii) a modified DNA that should act as a template during PCR. Using 5BrdUTP as an analog for TTP, we also observed the unwanted incorporation of dUTP into the resulting PCR fragment (the 5BrdUTP employed in the PCR synthesis was probably contaminated with a small amount of dUTP). In order to maximize the yield of the labeled DNA fragment and to reduce the observed amount of incorporated uridine we evaluated the substrate properties of four commercially available thermostable DNA polymerases: Taq from *Thermus aquaticus*, Pfu from *Pyrococcus furiosus* as well as two polymerase blends: Taq–Pwo and Taq–Pfu.

The first part of the current work shows the results of the screening of polymerases employed in the PCR reaction. Then the PCR protocol itself is described (optimized for the best-working polymerase), and the actual synthesis of the 500 bp fragment is confirmed by gel electrophoresis. The degree of DNA substitution with 5BrdU is assayed with the enzymatic digestion of the PCR product followed by the HPLC analysis of the digested mixture. Additionally, the HPLC method is validated and its performance checked against short, chemically synthesized oligonucleotide fragments labeled with 5BrdU. Finally, the sensitivity of the labeled PCR product to 300 nm photons is studied and its damage demonstrated with the help of gel electrophoresis and HPLC analysis coupled to the enzymatic digestion of the PCR product.

2. Materials and methods

2.1. Chemicals and reagents

The deoxyribonucleosides, DNase I and snake venom phosphodiesterase (SVP), were purchased from Sigma Aldrich, the bacterial alkaline phosphatase (BAP), the DNA purification kit and the DNA polymerases OptiTaq DNA, Taq and Pfu from Eurx Molecular Biology Products (Gdansk, Poland), and Marathon DNA polymerase from A&A Biotechnology (Gdynia, Poland). The PCR primers were obtained from Genomed (Warsaw, Poland), HPLC grade acetonitrile and formic acid from PoCh (Gliwice, Poland), and ethanol from Eurochem BGD (Tarnow, Poland). Ultrapure water was generated from a Milli-Q system from Hydrolab (Polska HLP).

2.2. Screening of polymerases for the incorporation of 5BrdUTP

Four kinds of commercially available, thermostable polymerases or polymerase blends were tested: Marathon DNA polymerase (Taq–Pwo blend), OptiTaq DNA polymerase (Taq–Pfu blend), Taq DNA polymerase and Pfu DNA polymerase. The amplification reactions were performed in 50 μl of a reaction mixture containing 5 ng of pUC19 template DNA, 0.5 μM of each primer, the appropriate concentration (depending on the type of polymerase) of polymerase, a reaction buffer supplied with enzyme (at 1× concentration) and 200 μM of triphosphates. Reactions containing natural nucleoside triphosphates dATP, dGTP, TTP and dCTP were used for a positive control. Reactions containing dATP, dGTP and dCTP (water was added instead of TTP) were used for a negative control. For all amplifications the standard cycle conditions were as follows: an initial denaturation step of 1.5 min at 94 °C, followed by 40 cycles of 20 s denaturation at 94 °C, 30 s annealing at 55 °C and 50 s elongation at 72 °C. The amplified region of pUC19 was 500 bp DNA with 50% GC content.

2.3. PCR reaction with 5BrdUTP

PCR reactions were performed using an Eppendorff thermocycler in 50 μl of a reaction mixture containing Tris–HCl (pH 9.0; 50 mM), $(NH_4)_2SO_4$ (20 mM), $MgCl_2$ (2.5 mM), 5% DMSO, 5 ng pUC19 plasmid DNA, 0.5 μM of each primer (the forward primer sequence: 5′-CTTAACTATGCGGCATCAGAGCA-3′ and the reverse primer sequence: 5′-ATTAATGCAGCTGGCACGACAG-3′), 200 μM of each dNTP (either dATP, dGTP, dCTP, TTP or dATP, dGTP, dCTP, 5BrdUTP) and 1 unit Taq–Pwo DNA polymerase blend (Marathon DNA polymerase), enriched with thermostable UTP-ase, which removes dUTP. The cycling conditions were as follows: an initial denaturation step of 1 min at 93 °C (not necessary), followed by 40 cycles of 30 s denaturation at 93 °C, 30 s annealing at 55 °C and 40 s elongation at 68 °C. The resulting PCR product was 500 bp in length. The synthesized DNA fragment was purified using the PCR/DNA Clean up purification kit (Eurx, Poland) and analyzed using agarose gel electrophoresis.

2.4. Agarose gel electrophoresis

1.5% or 2.0% agarose gels were prepared in TBE buffer [21]. The gels were visualized after staining with ethidium bromide using a 312 nm UV transilluminator.

2.5. Polyacrylamide gel electrophoresis

10% native and denaturing polyacrylamide gels were prepared in 1× TBE buffer [22]. The gels were visualized after staining with Sybr Green I using a 312 nm UV transilluminator and photographed with a SYBR Green gel stain photographic filter.

2.6. Enzymatic digestion

The purified DNA was digested to nucleosides by the simultaneous action of DNase I, snake venom phosphodiesterase (SVP) and bacterial alkaline phosphatase (BAP). 40 units of DNase I, 1 unit of BAP and 0.01 units of SVP were incubated with 12 μg of the purified 500 bp PCR fragment in a final volume of 100 μl in the following reaction buffer: Tris–HCl (pH 8.5; 10 mM), $MgCl_2$ (10 mM), $CaCl_2$ (1 mM) and DTPA (diethylenetriamine pentaacetic

Table 1
Summary of validation: linearity (L), limit of detection (LOD), limit of quantification (LQC) and relative standard deviation (RSD).

Nucleoside	L [μM]	LOD [μM]	LOQ [μM]	RSD [%]
dC	0.0028–1.4	0.114	0.342	1.91
dU	0.0029–1.4	0.057	0.171	1.62
dA	0.002–1.2	0.078	0.234	1.90
dG	0.002–1.0	0.124	0.372	1.73
dT	0.0025–1.2	0.072	0.216	1.88
5BrdU	0.0021–1.0	0.215	0.645	1.75

acid, Sigma Aldrich) (40 μM). The enzymatic digestion was performed at 37 °C for 3 h (1 h with each enzyme, sequentially). After digestion, reaction mixtures were purified by centrifugation using Microcon centrifugal filter units with a YM-3 membrane, NMWCO 3 kDa (Millipore). The resulting samples were dried down by rotary evaporation in a Speed-Vac Concentrator and dissolved in 60 μl of ultrapure water. 20 μl of the filtered sample was subjected to HPLC analysis.

2.7. HPLC analysis

The HPLC separation was performed on a Waters® 600E Delivery System with a Waters® 2487 Dual Lambda Absorbance detector, which was set at 260 nm for monitoring the effluents. A Waters® Atlantis reverse-phase dC_{18} column (4.6 mm × 150 mm; 5 μm in particle size and 100 L in pore size) with a mobile phase consisting of deionized water, acetonitrile (Sigma-Aldrich, Poland) and 1% formic acid (POCH S.A., Poland) (pH 2.55; 87.7:2:10.3, v/v/v) was used. The flow rate was set at 1 ml/min.

2.8. Linearity, precision, and accuracy of the HPLC protocol

Calibration curves were generated from the analysis of six different concentrations (0.002–1.5 mM) of deoxynucleosides. The calibration curves for each nucleoside were linear in the studied range of concentrations with a correlation coefficient of 0.99 or more. Limits of detection (LODs), limits of quantification (LOQs) and the assay precision (expressed as % RSD) are shown in Table 1.

2.9. Assessment of the enzymatic digestion/HPLC method for a short model sequence

The performance of the enzymatic digestion/HPLC method was verified for a short chemically synthesized oligonucleotide of the 5′-GCATCATCTCGACCATACCG-3′ sequence. Since the nucleosides have very different molar extinction coefficient one has to compare their integrated HPLC peaks with those of appropriate standards of known concentration for a quantitative analysis. The base composition of the oligonucleotide was calculated from the integrated HPLC signals corresponding to the particular nucleosides in the digested sample [23], namely, a mixture of known-concentration nucleoside standards was run prior to the HPLC analysis of the digested oligonucleotide. Comparison of the peak areas for the nucleoside mixture with the corresponding peaks areas in the digested sample allowed the exact concentrations of nucleosides in the digested oligonucleotide to be determined. Table 2, which summarizes the results obtained, demonstrates that the measured base composition (found) in the 20-nt oligonucleotide agrees well with the expected one (actual).

2.10. Irradiation

Prior to irradiation a DNA solution was freshly prepared and deoxygenated by purging with 99.998% pure argon for 5 min. Photolysis was carried out in quartz capillaries (3 mm × 3 mm) filled with the DNA solution in a total volume of 50 μl with a 200 W high-pressure mercury lamp for 60 min. During low temperature irradiations the capillaries were thermostated in a water-ice bath. The 300 nm wavelength of incident light (half-width 2.5 nm) was selected using a prismatic monochromator (SPM-2 Carl Zeiss, Jena).

Table 2
Base composition of a 20-nt oligonucleotide ($A_5G_3C_8T_4$) obtained with enzymatic digestion followed by HPLC separation.

Nucleoside	Measured	Actual	Deviation [%][a]
dC	7.86	8	1.75
dA	5.00	5	0.00
dG	3.06	3	2.00
dT	4.08	4	2.00

[a] Deviation = |actual − measured|/actual.

3. Results and discussion

3.1. Optimization of PCR reaction

We studied the ability of 5BrdUTP to act as a substrate in place of thymidine triphosphate (TTP) for thermostable DNA polymerases under typical PCR conditions. The following four commercially available thermostable polymerases were used in the current studies: Taq from *Therrmus aquaticus*, Pfu from *Pyrococcus furiosus.* as well as two polymerase blends: Marathon DNA polymerase (a mixture of Taq polymerase and Pwo polymerase from the hyperthermophilic archaebacterium *Pyrococcus woesei*, enriched with thermostable UTPase) and OptiTaq DNA polymerase (a mixture of Taq polymerase and Pfu polymerase). The amplified region on the pUC19 template was 500 bp DNA with 50% GC content. The PCR products were resolved by 1.5% agarose gel electrophoresis in TBE buffer (see Fig. 1). It was found that all the DNA polymerases tested could accept 5BrdUTP as a substrate (see Fig. 1, lanes 3, 6, 9, 12). The polymerases incorporated 5BrdUTP almost as efficiently as TTP into the newly synthesized strands of amplified DNA. However, several differences in the purity of the 5BrdU-labeled DNA products and in the number of commercial units of enzyme necessary for the reaction were observed. In comparison to the other polymerases tested (see Fig. 1, lanes 6, 9, 12) the use of Marathon DNA polymerase blend significantly improved PCR efficiency, sensitivity and specificity by reducing (see Fig. 1, lane 3) or eliminating (see Fig. 2, lane 3) nonspecific amplification and primer artifacts created prior to or during thermal cycling. Moreover, even 1 commercial unit of Marathon DNA polymerase was able to amplify a considerable amount of the specific 5BrdU-labeled DNA product (see Fig. 1, lane 3). In contrast to Marathon DNA polymerase, the other polymerases required the addition of 2.5 units for OptiTaq or 5 units for the Pfu

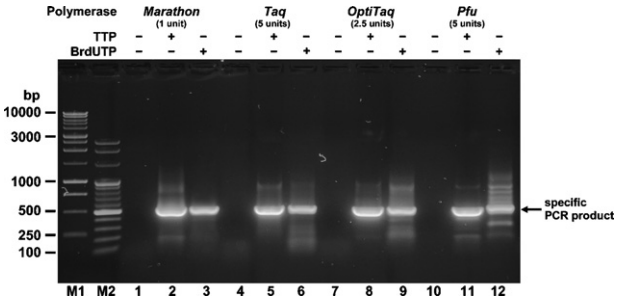

Fig. 1. The ability of different types of thermostable DNA polymerases to incorporate 5BrdU via PCR reactions. Lane M1: 1 kb DNA ladder (selected bands marked); lane M2: 100 bp DNA ladder; lanes 1–3: PCR reactions performed with Taq–Pwo polymerase blend; lanes 4–6: PCR reactions performed with Taq DNA polymerase; lanes 7–9: PCR reactions performed with Taq–Pfu polymerase blend; lanes 10–12: PCR reactions performed with Pfu polymerase; 1.5% agarose gel electrophoresis.

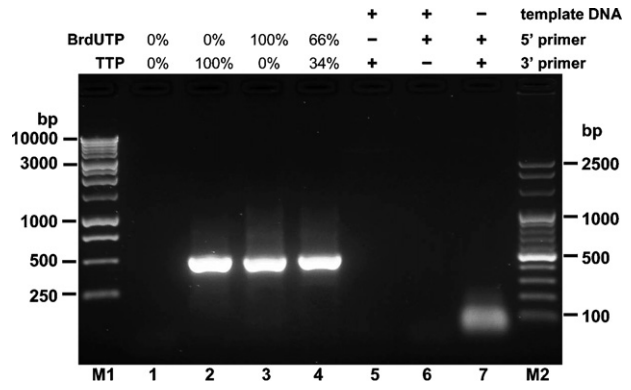

Fig. 2. Optimized 5BrdU incorporation into a PCR product using Marathon DNA polymerase. Lane M1: 1 kb DNA Ladder (selected bands marked); lane 1: PCR reaction without TTP or 5BrdU in the reaction mixture (negative control); lane 2: 500 bp PCR reaction product obtained in the presence of TTP (positive control); lane 3: 500 bp PCR reaction product obtained in the presence of 5BrdUTP; lane 4: 500 bp PCR reaction product obtained in the presence of a mixture of TTP/5BrdUTP; lanes 5–7: PCR control reactions; lane M2: 100 bp DNA ladder (selected bands marked). 1.5% agarose gel electrophoresis in TBE buffer.

and Taq DNA polymerases to obtain a comparable amount of the specific DNA product (see Fig. 1, lanes 6, 9, 12).

Considering the results described above, we finally chose Marathon DNA polymerase for the subsequent experiments. Because of the yield of the amplification product was higher than that of the other polymerases screened (not shown), the enzyme blend eventually selected enabled the cost of DNA synthesis to be reduced. The yield was further increased after the optimization of reaction conditions (see Fig. 2, lane 3): the temperatures of the denaturation and elongation steps were reduced slightly in comparison to the standard PCR reaction conditions. Marathon DNA polymerase also appeared to be more tolerant towards alterations in the conditions of the amplification reaction or the concentrations of reagents in the PCR mixture. These features make this enzyme more universal and suitable for producing 5BrdU-labeled DNA fragment regardless of the DNA template or primers used. Moreover, the presence of UTPase in the selected enzyme blend may effectively prevent the potential incorporation of uridine from UTP contaminated 5BrdUTP stocks.

The 100% 5BrdU labeled fragment of 500 bp was synthesized using the selected polymerase blend (see Fig. 2, lane 3). Depending on the ratio of thymidine to 5-bromouridine in the reaction mixture and the conditions of PCR, we were able to obtain a DNA product containing various amounts of the label (see Fig. 2, lanes 3 and 4). Fig. 3 shows a chromatogram depicting the result of enzymatic digestion of the 500 bp oligomer obtained (see Fig. 2, lane 3), in which all thymines (except those originating from DNA primers) were replaced with 5BrdU. The adenosine:5-bromouridine (1.04:1.00; see Table 3) and guanosine:cytidine (0.94:1.00; see Table 3) ratios calculated from the integrated HPLC signals show that the proposed analytical method possesses acceptable quantitative accuracy. The calculated nucleoside composition of the digested 500 bp fragment is demonstrated in Table 3. A similar approach, concerning the assay of 5-BrU [24,25] or 5-IU [13,26] in DNA fragments obtained from cells exposed to Hal-NBs, was published in the past. In summary, using various ratios of thymidine to 5-bromouridine we were able to control the degree of incorporation of the latter into the PCR products and then quantitatively assay the labeling efficiency. This PCR method mimics the situation present in real systems where, for instance, a cell is exposed to Hal-NBs during anti-cancer treatment. Thus, on the one hand we have worked out a relatively cheap method for synthesizing the Hal-NBs labeled oligomers with lengths not attainable using standard chemical methods of synthesis. On the other hand, the assay protocol described in this paper can be employed to analyze the genomic DNA originating from the cells cultured with Hal-NBs.

Table 3
Base composition of the 500 bp oligomer labeled completely with 5BrdU obtained by enzymatic digestion followed by HPLC separation.

Nucleoside	Measured	Actual	Deviation [%][a]
dC	265.62	263	0.99
dA	247.75	237	4.54
dG	249.39	263	5.18
5BrdU	237.24	237	0.10

[a] Deviation = |actual − measured|/actual.

3.2. Sensitivity of DNA substituted with 5BrdU to UV radiation

Several model studies employing short oligomers labeled with 1–2 molecules of 5BrdU demonstrated that irradiation of such modified DNA fragments with photons of ca. 300 nm leads to their damage: strand breaks (direct or indirect) and/or cross-links [11,12]. Relatively recently, the Sugiyama group reported that a significantly longer DNA, a 450 bp fragment, in which all thymines were substituted with 5BrdU, also develops strand breaks when irradiated with 302 nm at 0 °C [27]. Interestingly, these strand breaks were observed only when the labeled oligomer was heated at 95 °C for 20 min. Since in our PCR synthesized 500 bp fragment labeled with 5BrdU almost all thymines are substituted with 5BrdU, one should expect its enhanced photoreactivity. In order to confirm this hypothesis we carried out several irradiations enabling the photosensitivity of the labeled and non-labeled fragments to be compared. Thus, two solutions containing the labeled and

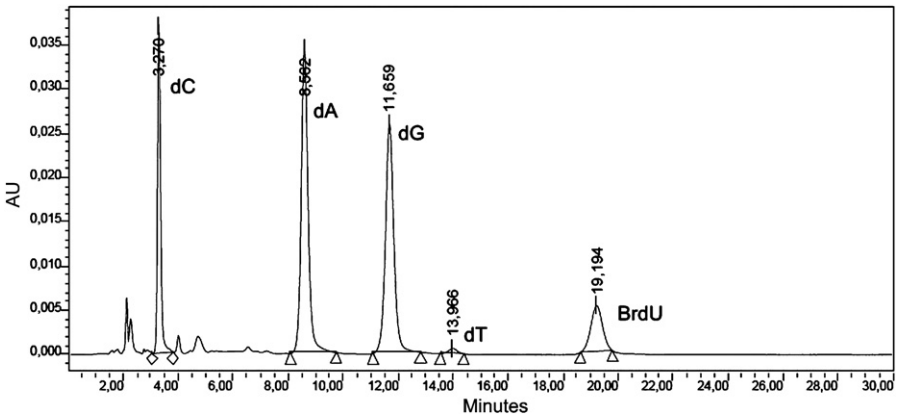

Fig. 3. Chromatogram of 5BrdU labeled 500 bp oligomer after enzymatic digestion.

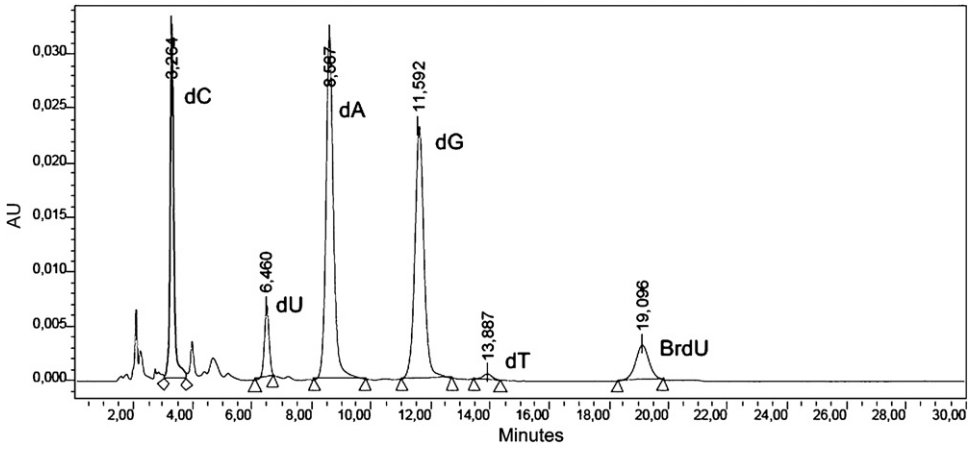

Fig. 4. Chromatogram of irradiated 5BrdU-labeled 500 bp oligomer after enzymatic digestion (ambient temperature).

non-labeled 500 bp oligomer at a concentration of ca. 0.3 mM of nucleobases were irradiated at ambient temperature for 1 h. Fig. 4 shows the chromatogram obtained after enzymatic digestion of the solution containing the labeled oligomer. Enzymatic digestion followed by HPLC analysis of the irradiated native (non-labeled) 500 bp oligomer yields a chromatogram identical to that obtained for the non-irradiated sample (see Fig. 3). However, as indicated by a comparison of Figs. 3 and 4, the UV irradiation of the labeled sample leads to a significant decrease in the amount of 5BrdU. Simultaneously, a new signal related to dU occurs. The ratio of the

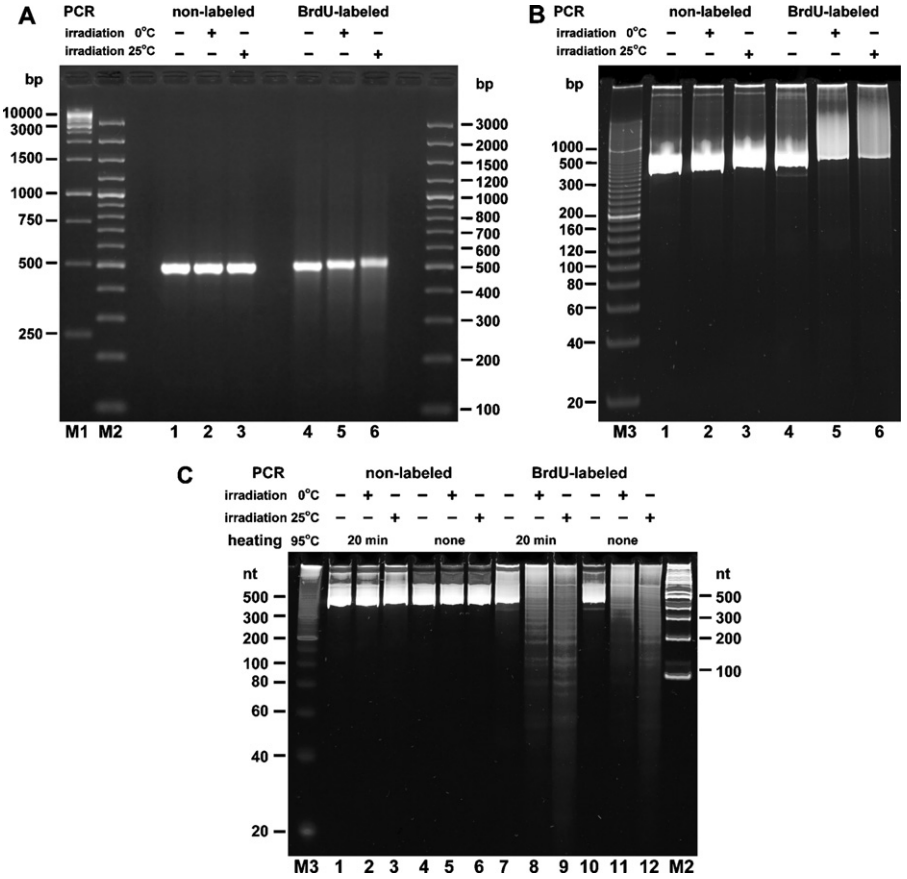

Fig. 5. The effect of irradiation on a 5BrdU-labeled 500 bp PCR fragment. (A) 2% Agarose gel electrophoresis in TBE buffer. Lane M1: 1 kb DNA ladder (selected bands marked); lane M2: 100 bp DNA ladder (selected bands marked); lane 1: non-labeled PCR fragment before irradiation; lane 2: non-labeled PCR fragment after irradiation at 0 °C; lane 3: non-labeled PCR fragment after irradiation at 25 °C; lane 4: 5BrdU-labeled PCR fragment before irradiation; lane 5: 5BrdU-labeled PCR fragment after irradiation at 0 °C; lane 6: 5BrdU-labeled PCR fragment after irradiation at 25 °C. (B) 10% polyacrylamide gel electrophoresis in TBE buffer. Lane M3: PCR 20 bp DNA ladder (selected bands marked); lane 1: non-labeled PCR fragment before irradiation; lane 2: non-labeled PCR fragment after irradiation at 0 °C; lane 3: non-labeled PCR fragment after irradiation at 25 °C; lane 4: 5BrdU-labeled PCR fragment before irradiation; lane 5: 5BrdU-labeled PCR fragment after irradiation at 0 °C; lane 6: 5BrdU-labeled PCR fragment after irradiation at 25 °C. (C). 10% denaturing polyacrylamide gel electrophoresis in TBE buffer. Lane M2: 100 bp DNA ladder (selected bands marked); lane M3: PCR 20 bp DNA ladder (selected bands marked). Samples from lanes 1 to 3 and 7 to 9 were incubated at 95 °C for 20 min. Lanes 1 and 4: non-labeled PCR fragment before irradiation; lanes 2 and 5: non-labeled PCR fragment irradiated at 0 °C; lanes 3 and 6: non-labeled PCR fragment irradiated at 25 °C; lanes 7 and 10: non-irradiated BrdU-labeled PCR fragment; lanes 8 and 11: BrdU-labeled PCR fragment irradiated at 0 °C; and lanes 9 and 12: BrdU-labeled PCR fragment irradiated at 25 °C.

5BrdU peak areas shown in Figs. 3 and 4 (irradiated:non-irradiated) amounts to 0.65 which demonstrates that after 1 h of irradiation ca. 35% of the initial amount of labeled DNA decomposes as a result of the photochemical process under scrutiny.

Although the enzymatic digestion of the irradiated DNA down to particular oligonucleotides enables the total yield of the photochemical process to be estimated, the information concerning the direct structural changes triggered by the UV photons in the labeled oligomer is lost. Therefore, we carried out additional experiments using agarose as well as native and denaturing polyacrylamide gel electrophoresis. Since within a similar study concerning a 450 bp 5BrdU labeled DNA fragment UV irradiation was carried out at 0 °C [27], we irradiated our samples at both 0 °C and the ambient temperature. Fig. 5A shows the electrophoregram that compares the migration of the irradiated and non-irradiated sample in the agarose gel. In contrast to the reference non-labeled samples (see Fig. 5A, lanes 1–3) that show no effect with irradiation and temperature, the bands for the irradiated labeled DNA (see Fig. 5A, lanes 5–6) are delayed with regard to the reference non-irradiated sample (cf. lane 4 with 5 and 6). Moreover, the delay is smaller for the sample irradiated at 0 °C (see Fig. 5A, lanes 5 and 6). Such an effect suggests that the UV-induced damage modifies the structure of double helix in such a way that the movement of DNA through the agarose gel is slowed down. Furthermore, as the delay effect is weaker at 0 °C, the secondary reactions which lead from the uridine-5-yl radical to strand breaks/cross-links are probably accompanied by substantial activation barriers that make the process of strand break formation temperature dependent. These conclusions are fully confirmed by the native polyacrylamide gel electrophoresis (see Fig. 5B). Again no irradiation and temperature effects are observed for non-labeled biopolymer (see Fig. 5B, lanes 1–3) whereas disappearance of the reference band and a distinctive smear in the higher-mass region appears for the irradiated labeled samples (see Fig. 5B, lanes 4–6). This effect is much more distinctive for the sample irradiated at the ambient temperature (Fig. 5B, cf. lanes 6 and 5). The smear observed in the higher-mass region suggests formation of both single strand breaks and cross-links as a result of irradiation. Yet another argument confirming the formation of strand breaks comes from denaturing PAGE. In contrast to native agarose or polyacrylamide gel electrophoresis, the latter technique enables all types of strand breaks (single, double and multiple) to be assayed. In Fig. 5C the result of denaturing PAGE electrophoresis for the non-irradiated labeled DNA (see Fig. 5C, lane 10) is compared with two other samples: irradiated at 0 °C (lane 11) and at the ambient temperature (lanes 12). Additionally, the non-labeled and labeled samples were heated for 20 min in 95 °C after irradiation as indicated by Ref. [27] (see Fig. 5C, lanes 2, 3, 8 and 9, respectively). For both irradiated samples a very long smear is observed, which indicates that UV radiation produced DNA fragments in a broad range of masses. Moreover, as indicated by Fig. 5C (cf. lanes 8 with 11 and 9 with 12), heating of the irradiated DNA makes the smear clearer and longer which suggests, in accordance with the findings of Ref. [27], that heat-labile sites are induced by irradiation. Finally, beside the smear a dozen or so distinctive bands are visible (Fig. 5C, lanes 8, 9, 11, and 12) which confirms the presence of "hot-spots" of increased UV sensitivity, being probably 5′-(G/C)AA^{B}rU^{Br}U-3′ and 5′-(G/C)A^{B}rU^{Br}U-3′ sequences [27].

4. Conclusions

In this paper a PCR protocol enabling a long DNA fragment to be completely labeled with 5BrdU was worked out. Using four commercially available DNA polymerases, we optimized the process of DNA synthesis. It turned out that Marathon DNA polymerase, which possesses UTPase activity (preventing the incorporation of uridine that could originate from the contamination of 5BrdUTP), leads to the highest yield of the desired PCR product.

The degree of 5BrdU incorporation into the synthesized DNA was assayed, after its total enzymatic digestion, with HPLC. Moreover, the HPLC method employed was validated by determining its linearity, limit of detection, limit of quantification and relative standard deviation for all assayed nucleosides. Although enzymatic digestion of DNA labeled with halouracils has been employed in the past, the details of the analytical protocol have not yet been characterized sufficiently [13,24–26,28–30].

The synthesized PCR product was digested with a mixture of DNase I, snake venom phosphodiesterase and bacterial alkaline phosphatase, and HPLC analysis confirmed that all thymines (besides those which originated from primers) were substituted with 5BrdU. Furthermore, the actual amounts of particular nucleosides corresponded pretty well to those calculated from the known sequence of the PCR product.

Aqueous solutions of synthesized DNA were irradiated with photons of 300 nm at 0 °C and ambient temperature, and the photolytes were analyzed by agarose and PAGE electrophoresis as well as by HPLC coupled to enzymatic digestion. Our results demonstrate unequivocally the photosensitivity of the labeled PCR product: namely, HPLC analysis showed a significant decrease in the signal related to 5BrdU and the occurrence of a new peak related to dU. On the other hand, native gel electrophoresis indicated the formation of strand breaks and cross-links, whereas the denaturing polyacrylamide gel electrophoresis demonstrates the formation of a significant number of strand breaks.

The PCR protocol presented in this work seems to be a general means of labeling DNA with any haloderivative of a nucleobase. PCR syntheses of DNA fragments labeled with 5-bromocytidine, 8-bromoadenosine and 8-bromoguanosine followed by studies of their photo- and radiosensitivity are under way in our laboratory.

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