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Bleomycin DNA damage: Anomalous mobility of 3′-phosphoglycolate termini in an automated capillary DNA sequencer

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ABSTRACT

An automated capillary DNA sequencer with laser-induced fluorescence detection can be utilised for DNA fragment analysis. The precise mobilities of DNA fragments with different chemical termini are especially important in the determination of the sequence specificity of DNA damaging agents. The aim of this study was to examine the electrophoretic mobility profile of DNA fragments with different 3′-termini. The nature of the 3′-teminal residue was found to have a major effect on the electrophoretic mobility of the DNA fragment, especially for 3′-phosphoglycolate termini that migrated anomalously by 3–6 nucleotides. Using the automated capillary sequencer, the electrophoretic mobilities of DNA fragments with different 3′-termini including 3′-hydrogen, 3′-hydroxyl, 3′-phosphate, and 3′-phosphoglycolate were extensively quantified and compared relative to each other. The 3′-hydrogen termini were generated by dideoxy sequencing; 3′-hydroxyl ends by minus sequencing; 3′-phosphate by Maxam–Gilbert chemical sequencing; and 3′-phosphoglycolate by bleomycin cleavage. The mobilities of these DNA fragments with different 3′-termini were found to be: (slowest) 3′-hydroxyl < 3′-hydrogen < 3′-phosphate < 3′-phosphoglycolate (fastest); with average relative mobilities of 0.00 < 0.12 < 0.63 < 4.42 nucleotides, respectively. The possible causes of the unusual electrophoretic mobility of the 3′-phosphoglycolate termini were discussed.

1. Introduction

The electrophoretic separation of DNA fragments can be accomplished with great precision utilising capillary gel electrophoresis [1]. The use of fluorescently-labelled DNA fragments combined with capillary electrophoresis (CE) and detection by laser-induced fluorescence (CE-LIF) has been widely utilised for DNA fragment analysis [2,3]. CE offers several advantages over slab gel electrophoresis in terms of speed, resolution, sensitivity, and data handling [3–10]. In CE, DNA fragments are separated as they migrate through a small-diameter capillary filled with an electrolyte and a polymer sieving matrix in the presence of high separating voltage [11]. The formation of DNA secondary structure is prevented by the presence of the denaturants urea and 2-pyrrolidinone in the electrolyte buffer and also by electrophoresis at a high running temperature [12]. It is preferable for DNA to be electrophoresed in a single-stranded form because it is able to interact more efficiently with the sieving matrix. The electrophoretic separation is based entirely on the size of the DNA since all of the fragments are single-stranded and hence, the DNA fragments are precisely ordered by molecular weight and can be accurately sized.

With most techniques involving DNA sequencing, the ends of the molecules are chemically identical and only differ by the number of nucleotides present in the analysed DNA fragments. However, for the analysis of the sequence specificity of DNA damaging agents, unusual chemical moieties can be formed at the ends of DNA molecules that can hinder analysis. For example, bleomycin cleavage of DNA produces 3′-phosphogycolate ends that migrate with an unusual mobility on capillary electrophoresis (see below).

Determining the sequence specificity of a DNA-damaging compound is important in elucidating its mechanism of action. To accomplish this technique, a ladder of molecular weight size markers e.g. those derived from dideoxy DNA sequencing reactions or Maxam–Gilbert chemical sequencing gel reactions, is required. It is also important to know the relative mobilities of DNA sequencing reaction products that end in 3′-hydrogen or 3′-phosphate, compared with the various 3′-terminal chemical moieties that can be formed with various DNA damaging agents [13].

Bleomycin is a glycopeptide antibiotic that is widely used as an anti-tumour drug [14–16]. The anti-tumour mechanism of action of bleomycin is thought to involve DNA-strand breaks

Abbreviations: CE, capillary electrophoresis; CE-LIF, capillary electrophoresis with laser-induced fluorescence detection; ddCTP, dideoxycytidine triphosphate; ddATP, dideoxyadenosine triphosphate; ddGTP, dideoxyguanosine triphosphate; ddTTP, dideoxythymidine triphosphate; LA, linear amplification.

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and bleomycin preferentially cleaves DNA at 5′-GT and 5′-GC dinucleotides [17–23]. Bleomycin cleavage generates a strand DNA break with 3′-phosphoglycolate and 5′-phosphate ends [24,25].

The aim of study was to examine the electrophoretic mobilities of DNA fragments bearing different 3′-terminal residues using the high resolution capability of CE-LIF. This aim arose from the observation that bleomycin cleavage of DNA resulted in 3′-phosphoglycolate terminated products that migrated anomalously when compared with the Maxam–Gilbert sequencing size markers. It has been documented that DNA fragments ending in a different chemical moiety migrate with a different mobility in high resolution polyacrylamide gel electrophoresis [24–28]. However, the effects of different terminal chemical identities on the mobility of DNA fragments have not been extensively examined in capillary electrophoresis. In this study, using CE-LIF with 5′-fluorescently labelled DNA, the electrophoretic mobilities of DNA fragments bearing different 3′-terminal residues including 3′-phosphate, 3′-hydroxyl, 3′-hydrogen and 3′-phosphoglycolate were compared. To this end, several sequencing techniques were employed. Dideoxy sequencing and minus sequencing were conducted using a 5′-fluorescently labelled primer to generate fragments bearing 3′-hydrogen and 3′-hydroxyl, respectively. In addition, a 5′-fluorescently labelled PCR product was subjected to different treatments including Maxam–Gilbert sequencing, restriction enzyme digests and bleomycin damage to produce DNA fragments bearing 3′-phosphate, 3′-hydroxyl and 3′-phosphoglycolate, respectively. The resulting DNA fragments were electrophoresed simultaneously on the ABI 3730 automated sequencer, and the data were analysed with GeneMapper software. The precise electrophoretic mobility profile of each fragment was determined and compared with the chemical identity of the 3′-terminus.

2. Materials and methods

2.1. Materials

Bleomycin was obtained from Bristol Laboratories, USA as the clinical preparation Blenoxane which contains approximately 70% bleomycin A_2 and 30% bleomycin B_2. Dideoxynucleoside triphosphates (ddNTPs) and deoxynucloside triphosphates (dNTPs) were purchased from Thermo Scientific. The REV oligonucleotide 5′-AACAGCTATGACCATG-3′ was from Invitrogen while the HPLC-purified FAM-SEQ oligonucleotide primer 5′-FAM-TCCCAGTCACGACGT-3′ was from Applied Biosystems. The restriction endonucleases *Eco*RI, *Dde*I, *Sma*I, *Sau3A*I, *Mbo*I and *Pvu*II were obtained from New England Biolabs; and *Alu*I and *Kpn*I were from Pharmacia Corp. Exonuclease IV was from New England Biolabs. Plasmid clone T7 contained 7 telomeric repeats, $(GGGTTA)_7$, in the vector pUC19 [29]. Plasmid DNA was purified using the QIAGEN Plasmid Midi kit.

2.2. Dideoxy sequencing

Dideoxy sequencing of DNA was carried out by the linear amplification procedure to produce DNA fragments bearing 3′-hydrogen termini [30,31]. The reactions employed the T7 plasmid digested with *Pvu*II as the template DNA [32]. In a 20 μl reaction, there was 1 mM either ddCTP, ddATP, ddTTP or ddGTP combined with 50 μM dNTP, 1 pmol FAM-SEQ, 40 ng T7 *Pvu*II-cut DNA, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8), 6.7 mM $MgCl_2$ and 1 U of *Taq* DNA polymerase. This reaction was subjected to 15 cycles of 95 °C for 30 s, 55 °C for 1 min, 72 °C for 1.5 min, followed by a final hold at 4 °C.

The dideoxy sequencing products were ethanol precipitated to remove artefact fragments [29]. A 10 μl aliquot was transferred to a new microcentrifuge tube containing 1 μl 3 M sodium acetate and 22 μl absolute ethanol and the solution was incubated on ice for 1 h, followed by centrifugation at 14,000 × *g* for 20 min. The supernatant was removed and the DNA pellet was washed twice with 70% (v/v) ethanol. The DNA sample was then dissolved in 10 μl of 10 mM Tris–HCl, pH 8.8, 0.1 mM EDTA.

2.3. Minus sequencing

Minus sequencing of DNA was carried out to produce DNA fragments bearing 3′-hydroxyl termini. It was performed by DNA linear amplification with limiting concentrations of one of the four dNTPs [33]. For instance, the minus G sequencing was carried out by linear amplification with regular amounts of dATP, dTTP and dCTP, with a limiting concentration of dGTP. The reactions employed the T7-*Pvu*II digest as the template DNA. Four types of reaction were carried out differing by the limiting nucleotide triphosphate. For the minus G sequencing, in a 20 μl reaction there was 0.75 μM dGTP, 0.3 mM each of dATP, dCTP and dTTP, 1 pmol FAM-SEQ, 40 ng T7 *Pvu*II-cut DNA, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8), 6.7 mM $MgCl_2$, 0.3 mM dNTPs and 1 U of *Taq* DNA polymerase. This reaction was thermally cycled using the same PCR parameters described in the dideoxy sequencing method. For the other minus sequencing reactions (A, T, C), the same conditions of the reactions were used except that the limiting deoxynucleoside triphosphate concentrations were 0.75 μM dCTP, 5 μM dATP or 5 μM dTTP. The minus sequencing products were ethanol precipitated as above, to remove artefact bands.

2.4. Preparation of the 5′-FAM-labelled T7 PCR product

A 130 bp T7 PCR product, fluorescently-labelled at the 5′-end, was obtained by conducting a polymerase chain reaction (PCR) on the T7 plasmid with a 5′-fluorescently labelled forward primer (FAM-SEQ) and a non-labelled reverse primer (REV). In a total volume of 20 μl, there was 3.48 ng of plasmid T7, 10 pmol FAM-SEQ, 10 pmol REV, 16.6 mM $(NH_4)_2SO_4$, 67 mM Tris–HCl (pH 8.8), 6.7 mM $MgCl_2$, 0.3 mM dNTPs, and 1 U of *Taq* DNA polymerase. This mixture was thermally cycled with the following parameters: 95 °C for 5 min, 30 cycles of 95 °C for 45 s, 57 °C for 1 min, 72 °C for 1.5 min, an extension at 72 °C for 10 min, and a hold at 4 °C. The FAM-labelled PCR products were then purified on a 10% (w/v) native polyacrylamide gel, ethanol precipitated, and dissolved in 25 μl 10 mM Tris–HCl, pH 8.8, 0.1 mM EDTA.

2.5. Restriction enzyme digest

The restriction enzyme digest was prepared by mixing approximately 18 ng of the fluorescently-labelled T7 PCR product with 10× restriction enzyme buffer (as supplied by the manufacturer) followed by the addition of 1 μl restriction enzyme (20 U/μl) in a total volume of 10 μl and reaction was incubated at 37 °C for 1 h.

2.6. Maxam–Gilbert G + A cleavage

Maxam–Gilbert G + A cleavage of the fluorescently-labelled PCR product was carried out as previously described by Belikov and Wieslander [34]. Briefly, the reaction was prepared by mixing 10 μl of a solution of 3% (v/v) diphenylamine in formic acid with 5 μl containing 24 ng of the fluorescently-labelled PCR product and 144 ng of chicken DNA as carrier. The reaction was incubated at 37 °C for 1 h and 100 μl 0.3 M sodium acetate (pH 5.5) was then added. The mixture was extracted three to four times with water-saturated ether. The DNA was ethanol precipitated and redissolved in 10 μl of 10 mM Tris–HCl, pH 8.8, 0.1 mM EDTA.

2.7. Bleomycin treatment

In a total volume of 10 μl, there was 12 ng of the fluorescently-labelled PCR product, equal concentrations of $FeSO_4$ and bleomycin (0.03–0.2 mM), and 720 ng of purified chicken DNA as carrier [23]. The reaction was incubated at 37 °C for 30 min, followed by ethanol precipitation and was redissolved in 10 μl of 10 mM Tris–HCl, pH 8.8, 0.1 mM EDTA.

2.8. Treatment with endonuclease IV

The bleomycin-cleaved products were treated with endonuclease IV to convert the 3′-phosphoglycolate termini to 3′-hydroxyl ends [28]. In a total volume of 10 μl, there was 2 μl of the FAM-labelled bleomycin-treated DNA that was incubated with 5 U endonuclease IV (NEB) at 37 °C for 1 h in 1× NEBuffer 3 (50 mM Tris–HCl pH 7.9, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol) as supplied by the manufacturer. The 10 μl reaction was then ethanol precipitated and dissolved in 10 μl of 10 mM Tris–HCl, pH 8.8, 0.1 mM EDTA.

2.9. Capillary electrophoresis with laser-induced fluorescence

From each of the procedures, 2 μl of FAM-labelled DNA was sent to the Clive and Vera Ramaciotti Centre for Gene Function Analysis to be processed on the ABI 3730 automated capillary sequencer. The sample was mixed with a 20 μl mixture of LIZ500 marker and HiDi formamide (10 μl LIZ500 in 1 ml HiDi). After placing a 15 μl aliquot of this solution into a 96-well plate, it was denatured at 95 °C for 5 min, injected at 1.6 kV for 15 s into the automated sequencer, and electrophoresed for approximately 32 min at 63 °C. Fluorescence was measured after excitation by an argon ion gas laser at 488/514.5 nm and detection at 540 nm. These data were analysed with GeneMapper software version 3.7 (Applied Biosystems) using a modified AFLP algorithm [29].

3. Results

3.1. Bleomycin damage of the 5′-FAM-labelled T7 PCR product

A section of the plasmid T7 containing seven telomeric repeat sequences, $(GGGTTA)_7$, (Fig. 1) was amplified by PCR with FAM-SEQ and REV oligonucleotides as primers to generate the 5′-FAM-labelled T7 PCR product. The purified 5′-FAM-labelled T7 PCR product was damaged with bleomycin to generate DNA fragments bearing 3′-phosphoglycolate residues. The products were electrophoresed on the automated capillary sequencer alongside DNA sequencing reactions to examine the anomalous migration of bleomycin-induced DNA fragments bearing 3′-phosphoglycolate termini. The resulting data were analysed with the GeneMapper software and illustrated as electropherograms (Fig. 2).

Fig. 2 shows the pattern of cleavage of the 5′-FAM-labelled T7 PCR product caused by bleomycin treatment. The level of background was determined by the no-drug control and was found to be low in each experiment, allowing bleomycin damage to be clearly observed. The evenly spaced series of 6 peaks labelled as T1–T6 in Fig. 2A represents bleomycin damage in the telomeric repeat sequences of the 5′-FAM-labelled T7 PCR product.

The 5′-FAM-labelled T7 PCR product was subjected to Maxam–Gilbert G + A sequencing (Fig. 2B). The sequence specificity of bleomycin cleavage was assessed by superimposing the bleomycin damage electropherogram Fig. 2A onto the G + A sequencing (Fig. 2B) to give (Fig. 2C). In a previous paper [23], the DNA sequence specificity of telomeric bleomycin cleavage was determined with a 3′-FAM-labelled T7 PCR product and was found to be mainly at 5′-GT (with lesser amounts at 5′-GG). However, the bleomycin damage peaks (solid arrows in Fig. 2C) did not occur at the expected 5′-GT sites in the G + A sequencing (dashed arrows in Fig. 2C) due to the anomalous migration of DNA bleomycin cleavage fragments bearing 3′-phosphoglycolate ends. This anomalous mobility of the DNA fragments bearing the 3′-phosphoglycolate can be easily observed at the telomeric sequence in Fig. 2C. These main bleomycin peaks (solid arrows in Fig. 2C) should migrate with a mobility spacing of 6 nucleotides when compared with the G + A sequencing. In the first three repeats, the peaks T1–T3 lined up with the A nucleotide of 5′-tAGGGt repeats of the G + A sequencing rather than the Gt. The T4 peak moved to between the A and the G nucleotides, while T5 and T6 lined up with the first G nucleotide in 5′-tAGGGt. In general, the mobility of bleomycin damage products in the telomeric regions of the 5′-FAM-labelled T7 DNA fragment migrated faster than expected by 3–4 nucleotides.

3.2. Electrophoretic mobilities of DNA fragments bearing different 3′-terminal residues

To produce a series of DNA fragments fluorescently-labelled at a common 5′-end but differing solely by a 3′-terminal residue, two DNA templates were used: the T7 *Pvu*II-cut DNA fragment and the 5′-FAM-labelled T7 PCR product. Fig. 3 shows electropherograms of the dideoxy G and minus G sequencing which were representative of the complete sets of the four dideoxy and four minus sequencing reactions, respectively. The *Alu*I electropherogram exemplifies the data obtained from restriction enzyme digests of the 5′-FAM-labelled PCR product. The G + A sequencing and bleomycin damage of the PCR product are also shown.

The dideoxy sequencing of the T7 *Pvu*II-cut DNA fragment resulted in a pattern of peaks that agreed with the known sequence of the DNA fragment (Fig. 3). These terminated DNA sequences represent the group of DNA fragments bearing 3′-hydrogen termini.

Electropherograms derived from the minus sequencing of the T7 *Pvu*II-cut DNA fragment were compared with those of the dideoxy sequencing. Peaks generated from the minus sequencing displayed the same pattern as those from the dideoxy sequencing, but were displaced by one nucleotide to the left (shorter) (Fig. 3). For example, if dGTP was the limiting triphosphate (minus G sequencing), the peaks should be present one nucleotide before the ddG positions, due to lack of the dGTP in the minus G reaction. Peaks obtained from the electropherograms of the complete minus sequencing set covered the sequence of the T7 *Pvu*II-cut DNA fragment from base pair 28 to base pair 270. The terminated DNA fragments created by minus sequencing reactions represent the group of DNA sequences bearing 3′-hydroxyl termini.

The *Eco*RI, *Kpn*I, *Dde*I, *Sma*I, *Sau3A*I and *Mbo*I restriction enzymes have a single restriction enzyme site in the analysed T7 sequence and digests of the 5′-FAM-labelled T7 PCR product produced a single peak correlating with the single restriction enzyme sites in the T7 DNA sequence. *Alu*I has three restriction enzyme sites (Fig. 1) and an *Alu*I partial digest of the 5′-FAM-labelled T7 PCR product generated 3 different peaks which corresponded to the three *Alu*I sites in the DNA fragment (Fig. 3A). These restriction enzyme-digested PCR products contain 3′-hydroxyl ends and the resulting peaks should co-migrate with the corresponding peaks in the minus sequencing. Electropherograms of the restriction enzyme digests and minus sequencing were overlaid. The peaks derived from the restriction enzyme digests agreed precisely with those from the minus sequencing.

Electropherograms derived from linear amplification of the T7 plasmid digested with the *Eco*RI, *Kpn*I and *Dde*I restriction enzymes resulted in both single and double peaks. This is due to the *Taq* DNA polymerase extending the FAM-SEQ primer to the digested point and adding a non-templated A nucleotide to the 3′-end of the DNA strand [35]. Generally, the DNA polymerase added the A nucleotide

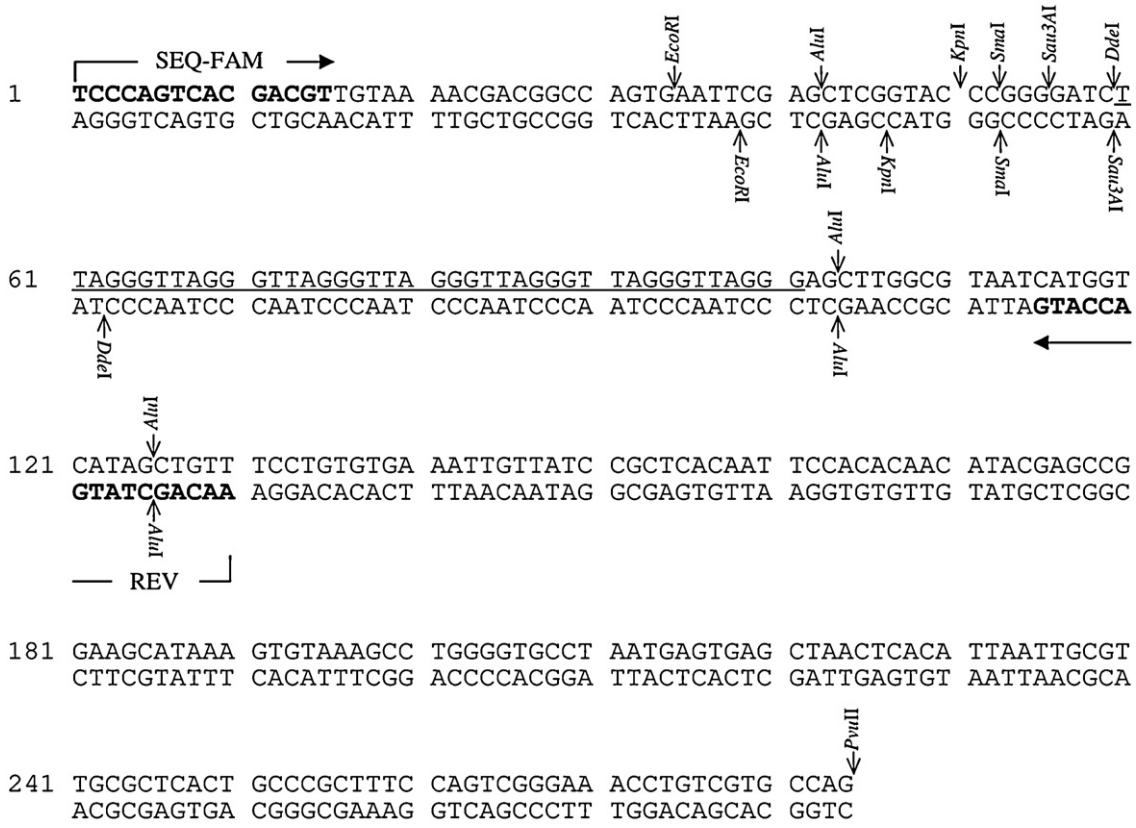

Fig. 1. The double-stranded sequence of the T7 *Pvu*II-cut DNA fragment. The upper strand is written 5′–3′ and the lower strand is written 3′–5′. The FAM-SEQ and REV oligonucleotide primers are in bold and the horizontal arrow heads represent the 5′–3′ direction of DNA polymerase extension. The seven telomeric sequences are underlined. The positions of restriction enzyme cleavage sites are labelled with vertical arrows.

to all of the linear DNA fragments generated by LA of the *Eco*RI and *Kpn*I digested T7 and approximately 56% in the linear fragments obtained from LA of the *Dde*I digested T7.

Maxam–Gilbert G + A sequencing and bleomycin damage of the 5′-FAM-labelled PCR product have been described above and are shown in Fig. 3. DNA fragments produced by G + A sequencing and bleomycin damage represent the group of DNA fragments bearing 3′-phosphate and 3′-phosphoglycolate, respectively. The G + A sequencing and bleomycin damage reactions destroy the indicated nucleotides as part of the chemical reaction that generates the cleavage site. Therefore, the actual DNA fragment size is one nucleotide shorter than the one annotated in the electropherograms. For example, the 5′-ttA sequence in the telomeric sequence of the T7 plasmid when subjected to G + A sequencing, the A nucleotide was labelled as the indicated nucleotide in the electropherogram; however, the A nucleotide is destroyed in the chemical process and the DNA fragment ends in 5′-tt. Hence, for G + A and bleomycin cleavage, the sequences in Fig. 3 have been adjusted to compensate for this effect.

In order to assess the relative mobilities of DNA fragments bearing different 3′-terminal residues, the GeneMapper size was compared with the expected size from the known DNA sequence. There is a discrepancy between the GeneMapper size and the expected size. This discrepancy was calculated by subtracting the GeneMapper size of a particular peak from its corresponding expected size, and plotted against the DNA fragment length (Fig. 4). Generally, the size discrepancy values of DNA fragments with different 3′-terminal residues showed similar fluctuating trends across the length of the DNA fragment (the *x*-axis). Dideoxy sequencing and minus sequencing covered the same range across the *x*-axis between bp 27 and 270, while G + A sequencing, bleomycin damage, restriction enzyme digests and LA of the digested DNA had a shorter coverage, between bp 26 and 125. This is because of the difference in the analysed length of the T7 *Pvu*II-cut DNA fragment and the FAM-SEQ-REV PCR product; with the former being 284 bp, and the latter 130 bp. Almost all the GeneMapper-derived sizes of DNA fragments were smaller than their expected size, except for the area between base pair 140 and 221 where most of the 3′-hydroxyl and 3′-hydrogen DNA fragments had greater experimental size than expected. The 3′-hydroxyl and 3′-hydrogen DNA fragments showed the most similar and smallest discrepancy relative to other 3′-ends, ranging between −1.13 and +3.57 nucleotides. The size discrepancy of the 3′-phosphate DNA fragments was slightly greater than those with the 3′-hydroxyl and 3′-hydrogen, ranging between 0.64 and 4.48 nucleotides. Of all the 3′-terminal ends, the 3′-phosphoglycolate displayed the greatest size discrepancy, ranging between 3.98 and 8.52 nucleotides. Since the size discrepancies of these DNA fragments have a direct relationship with their electrophoretic mobilities, it can be inferred that the gradient in the mobilities of DNA with different 3′-ends is in the following order: (slower) 3′-hydrogen = 3′-hydroxyl < 3′-phosphate < 3′-phosphoglycolate (faster). In addition, there were four inflection points of the curve which correlate with four bands of the LIZ-500 commercial size marker including those at 50 bp, 100 bp, 150 bp and 200 bp.

The mobilities of DNA fragments bearing 3′-hydrogen, 3′-phosphate, 3′-phosphoglycolate and restriction enzyme-derived 3′-hydroxyl were calculated relative to the minus sequencing-derived 3′-hydroxyl DNA fragments. The GeneMapper sizes of the DNA fragments with 3′-hydrogen, 3′-phosphate, 3′-phosphoglycolate and restriction enzyme-derived 3′-hydroxyl were subtracted from those of the minus sequencing-derived

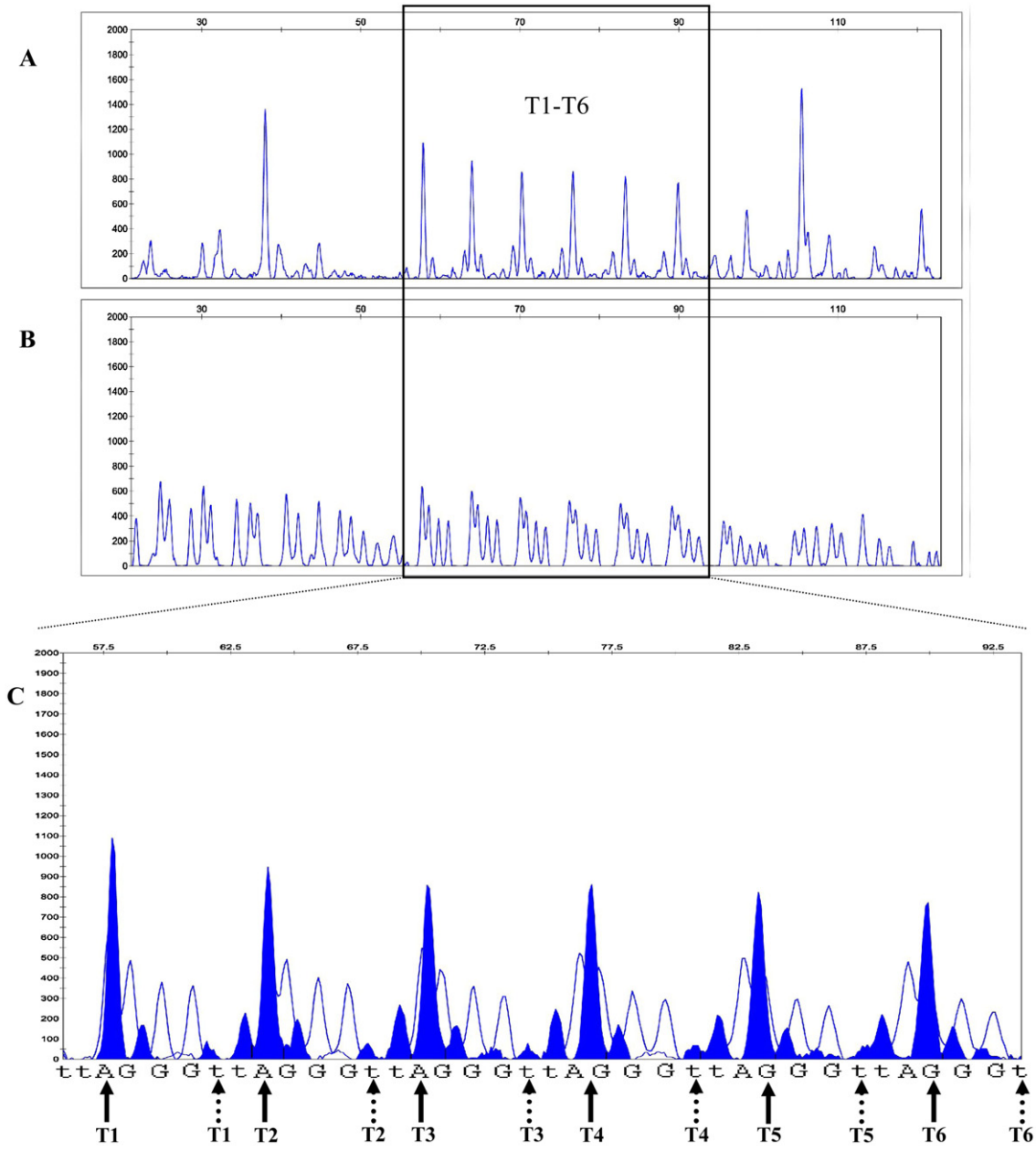

Fig. 2. Anomalous migration of DNA fragments derived from bleomycin cleavage of the 5′-FAM-labelled T7 PCR product. Electropherograms showing bleomycin cleavage (A) and Maxam–Gilbert G + A sequencing (B) of the 5′-FAM-labelled T7 PCR product. The areas in the box are bleomycin cleavage and G + A sequencing in the telomeric repeats T1–T6 which are superimposed in the bottom trace (C), with the filled and unfilled peaks being the bleomycin cleavage and G + A sequencing, respectively. The sequence is shown underneath based on the G + A sequencing and written 5′–3′. The black arrows T1–T6 represent the observed bleomycin cleavage sites in the telomeric sequence of the 5′-FAM-labelled T7 PCR product determined by comparison with the G + A sequencing. The dashed arrows T1–T6 represent the expected bleomycin damage sites in the telomeric sequence. The electropherograms depict the relative size of the DNA fragments (nucleotides) along the *x*-axis and the relative fluorescent intensity along the *y*-axis. Peaks appearing to the left of the electropherogram are shorter fragments and migrate faster than the larger fragments at the right of the electropherogram.

3′-hydroxyl DNA fragments. The relative mobility was than plotted against the DNA fragment size and shown in Fig. 5. The relative mobilities of DNA fragments with 3′-hydrogen and restriction enzyme-derived 3′-hydroxyl were the smallest and the most similar, relative to other 3′-ends, with the former ranging from −0.75 to +0.65 nucleotides (average 0.12 ± 0.01) and the latter from −0.19 to +0.19 nucleotides (average 0.00 ± 0.05). This indicates the similarity in electrophoretic mobility of these DNA fragments. The relative mobility of 3′-phosphate DNA fragments was slightly larger than the 3′-hydrogen and 3′-hydroxyl ranging from −0.39 to +1.66 nucleotides (average 0.63 ± 0.05), while that of 3′-phosphoglycolate DNA fragments were the largest ranging from +3.01 to +6.13 nucleotides (average 4.42 ± 0.27). As expected, the restriction enzyme-derived 3′-hydroxyl DNA fragments showed almost no difference with those of the 3′-hydroxyl generated by the minus sequencing, with the average relative mobility 0.00 ± 0.05 nucleotides. The relative mobility values of DNA fragments with different 3′-ends have a direct relationship with their electrophoretic mobilities. Therefore, the mobilities of these DNA fragments can be arranged following the order: (slowest) 3′-hydroxyl < 3′-hydrogen < 3′-phosphate < 3′-phosphoglycolate (fastest), correlating to the average relative mobilities of $0.00 < 0.12 < 0.63 < 4.42$ nucleotides, respectively.

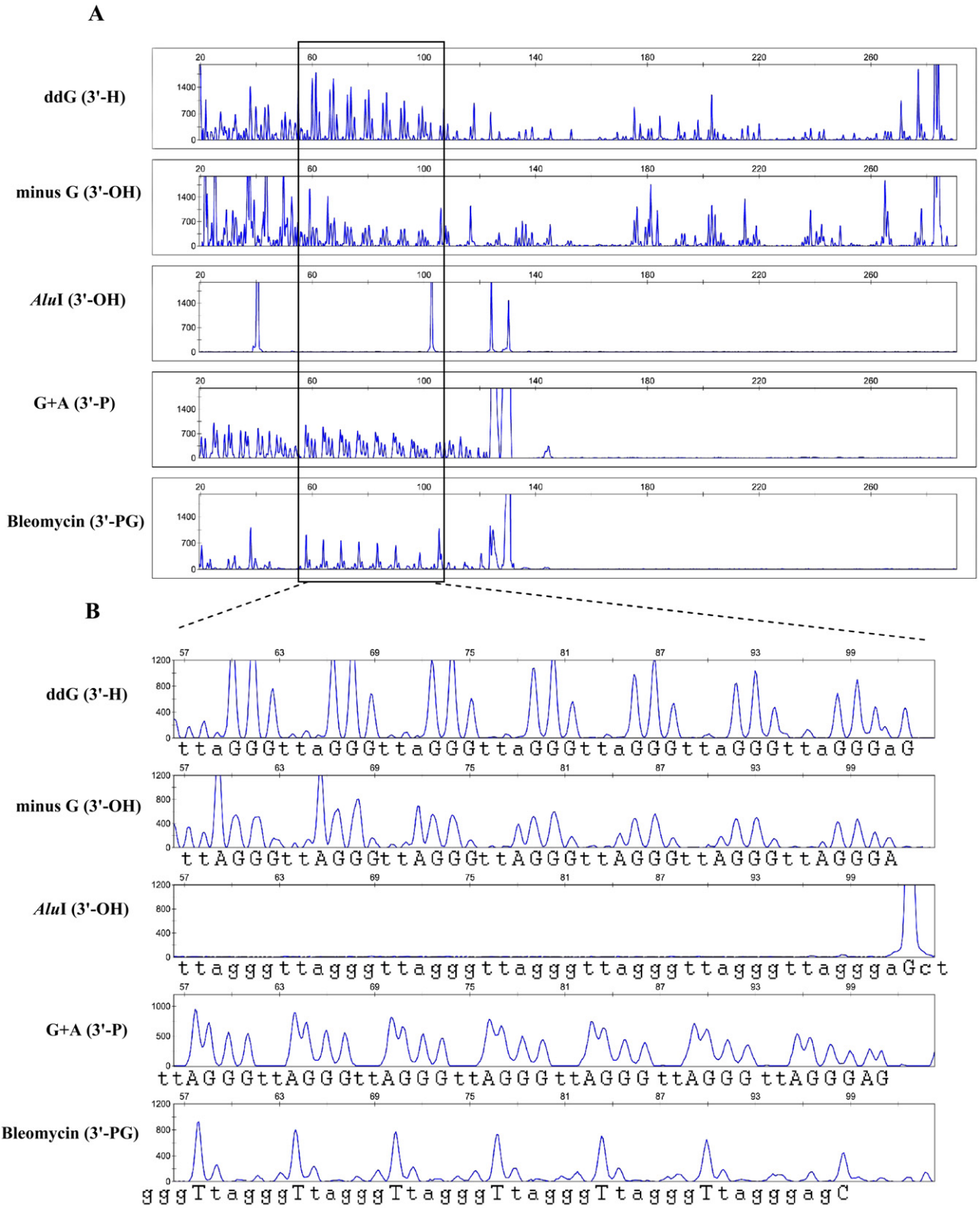

Fig. 3. Comparison of electrophoretic mobilities of the DNA fragments bearing different ends: 3′-hydrogen, 3′-hydroxyl, 3′-phosphate and 3′-phosphoglycolate. (A) Electropherograms showing dideoxy sequencing G and minus sequencing G of the T7 *Pvu*II-cut DNA fragment; *Alu*I digestion, G + A sequencing and bleomycin damage of the 5′-FAM-labelled T7 PCR product. The large peaks at the far right of the ddG and minus G electropherograms are the full length product at 284 bp; while the full length product of the 5′-FAM-labelled T7 PCR product is at 130 bp for the *Alu*I, G + A and bleomycin electropherograms. Each electropherogram is labelled with its corresponding 3′-terminal group. The DNA fragment size (nucleotides) is along the *x*-axis and relative fluorescent intensity along the *y*-axis. (B) The telomeric region is expanded and the corresponding sequences are written from 5′–3′ underneath the electropherograms. Note that the indicated nucleotides for the G + A sequencing and bleomycin cleavage in capital letters are destroyed, and hence one base pair needs to be deducted when these electropherograms are compared with those of the dideoxy sequencing G, minus sequencing G and restriction enzyme digests.

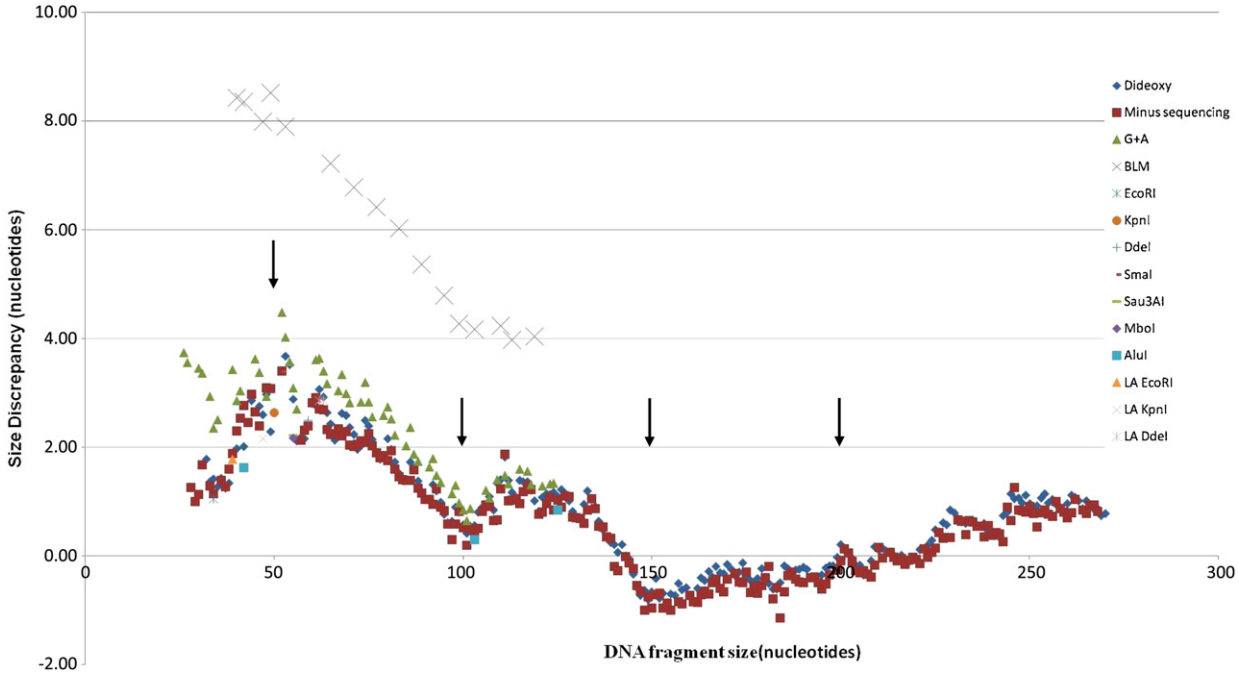

Fig. 4. Discrepancy between GeneMapper size and real fragment size. The discrepancy between the observed GeneMapper size and the expected fragment size is shown. The T7 *Pvu*II-cut DNA fragment was subjected to dideoxy sequencing and minus sequencing using the FAM-SEQ primer to generate DNA fragments bearing 3′-hydrogen and 3′-hydroxyl, respectively. Linear amplification with the same primer was also performed on the T7 plasmid digested with different restriction enzymes, including *Eco*RI, *Kpn*I and *Dde*I. The 5′-FAM-labelled T7 PCR DNA product (130 bp in length) was subjected to bleomycin cleavage, G + A sequencing and restriction enzyme digests (including *Eco*RI, *Kpn*I, *Dde*I, *Sma*I *Sau*3AI, *Mbo*I and *Alu*I) to generate fragments bearing 3′-phosphoglycolate, 3′-phosphate and 3′-hydroxyl, respectively. All DNA fragments were electrophoresed on the automated sequencer simultaneously and the resulting data were analysed with the GeneMapper software. The DNA fragment sizes calculated by GeneMapper were subtracted from the known fragment sizes to determine the discrepancy between the GeneMapper and expected sizes. The *x*-axis is the known DNA fragment size, while the *y*-axis is the size discrepancy between the GeneMapper size and real fragment size. The arrows represent the inflection points of the curve that correlate with four bands of the LIZ-500 commercial size marker (50 bp, 100 bp, 150 bp and 200 bp).

The bleomycin-treated DNA was subjected to a treatment with endonuclease IV to remove the 3′-phosphoglycolate terminus and produce a 3′-hydroxyl terminus. The mobility of the bleomycin-cleaved fragments decreased by approximately 3–6 nucleotides after endonuclease IV treatment (Fig. 6) and migrated at the position expected of a DNA fragment with a 3′-hydroxyl terminus (average -0.01 ± 0.06 nucleotides, range -0.11 to $+0.10$). Hence it is likely that the anomalous mobility of the bleomycin-cleaved DNA is due to the presence of 3′-phosphoglycolate ends [28].

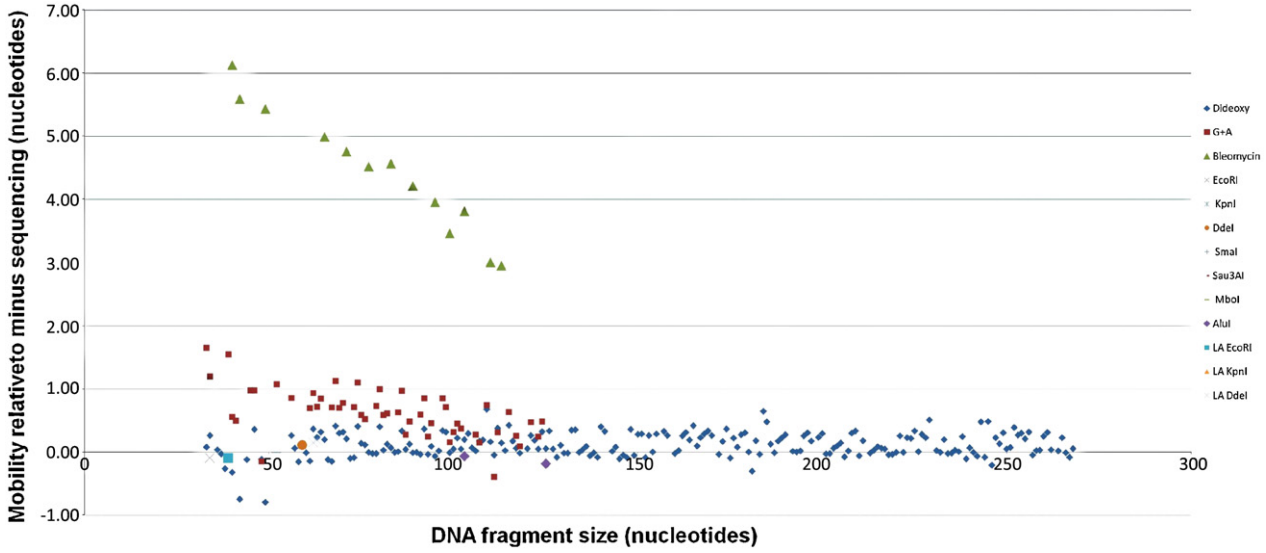

Fig. 5. The relative electrophoretic mobilities of DNA fragments bearing 3′-hydrogen, 3′-phosphate, 3′-phosphoglycolate and restriction enzyme-derived 3′-hydroxyl. The electrophoretic mobilities of DNA fragments bearing 3′-phosphate, 3′-hydrogen, 3′-phosphoglycolate and restriction enzyme-derived 3′-hydroxyl were compared with 3′-hydroxyl-ending DNA fragments derived from minus sequencing. The relative mobilities were calculated by subtracting the GeneMapper sizes of the DNA fragments with 3′-hydrogen, 3′-phosphate, 3′-phosphoglycolate and restriction enzyme-derived 3′-hydroxyl termini from those of the minus sequencing-derived 3′-hydroxyl DNA fragments and plotted against the known DNA fragment size. The *x*-axis is the known DNA fragment size, while the *y*-axis is the mobility relative to minus sequencing (nucleotides) of the DNA fragments with different 3′-ends.

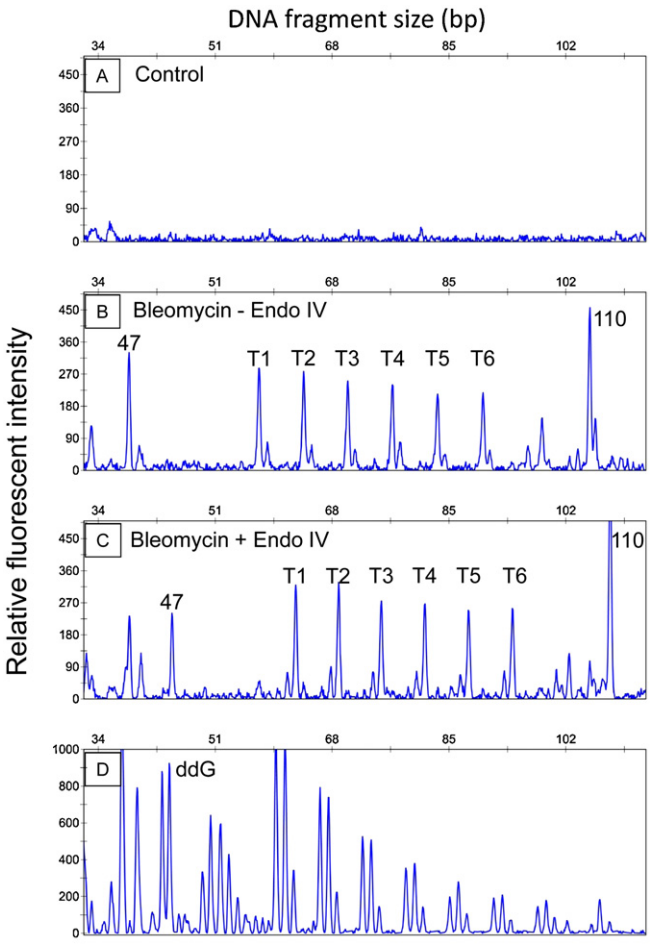

Fig. 6. An electropherogram depicting the effect of endonuclease IV treatment on bleomycin-cleaved DNA. The 5′-FAM-labelled T7 PCR product was reacted with bleomycin to produce 3′-phosphoglycolate termini and subsequently treated with endonuclease IV to convert the termini to 3′-OH ends. (A) Blank control containing no bleomycin; (B) treatment with 0.10 mM bleomycin but not endonuclease IV; (C) treatment with 0.10 mM bleomycin and endonuclease IV; (D) the dideoxy G sequencing track. T1–T6 indicate the position of the bleomycin cleavage sites in the telomeric repeat; while other bleomycin cleavage sites, 47 and 110, are labelled with their position in bps. The electropherograms depict the relative size of the DNA fragments (nucleotides) along the *x*-axis and the relative fluorescent intensity along the *y*-axis.

4. Discussion

The separation of single-stranded DNA fragments by capillary electrophoresis mainly depends on the length of the fragment [11]. The identity of the 3′-terminus of the DNA fragment is also a crucial factor in the determination of its mobility and separation [13,36]. The bleomycin cleavage of DNA generates a 3′-phosphoglycolate terminal group as well as a 5′-phosphate [18,37]. The migration of the 3′-phosphoglycolate species has been observed on polyacrylamide sequencing gels, but has not been previously documented for capillary sequencing gels. The aim of this study was to examine the anomalous migration of DNA fragments with 3′-phosphoglycolate termini on capillary sequencing gels and extensively quantify their electrophoretic mobility compared with several other terminal groups including 3′-phosphate, 3′-hydrogen and 3′-hydroxyl. DNA capillary electrophoresis is known to have a greater base-pair resolution than polyacrylamide gel electrophoresis, and thus provided a more precise profile of DNA fragment electrophoretic mobility.

4.1. Discrepancy with the GeneMapper-determined size

There was a discrepancy between the GeneMapper-determined size and the true fragment size. This was due to the commercial size marker having a different sequence and fluorophore from the analysed DNA fragment [13,38]. This discrepancy was studied in detail using DNA fragments containing the same number of nucleotides but differing only by the chemical group at the 3′-end (Fig. 4). The level of discrepancy clearly depended upon the length of the DNA fragment and the type of 3′-termini. The discrepancy was the greatest for DNA fragments approximately 50 bp long. One of the intriguing points in Fig. 4 is the four inflection points of the discrepancy curve that correlated with the sizes of four bands in the commercial size marker. The DNA fragments in the LIZ-500 commercial size marker are used in the algorithm to estimate the size of the analysed fragment and this results in errors when estimating the size of DNA fragments [39]. This is in agreement with a study by Akbari et al. using the same automated sequencer and commercial size marker who also found similar sizing discrepancies [40].

4.2. Electrophoretic mobilities of DNA fragments bearing different 3′-terminal residues

The mobility discrepancy between 3′-hydroxyl and 3′-hydrogen DNA fragments was very small (0.12 nucleotides). This is in agreement with previous studies using both polyacrylamide and capillary electrophoresis systems [41–46]. Dideoxy sequencing products with 3′-hydrogen ends have been used to map sites of DNase I digestion that generate DNA fragments with 3′-hydroxyl, on both polyacrylamide and capillary electrophoresis system [46,47]. There was no discrepancy in electrophoretic mobilities of DNA fragments generated by dideoxy sequencing and DNase I digestion reported in these studies. It is interesting to note that, due to the higher resolving power of capillary electrophoresis compared with polyacrylamide gel electrophoresis, the difference in electrophoretic mobilities between DNA fragments with 3′-hydrogen and 3′-hydroxyl by 0.12 nucleotides, could be detected with the automated sequencer in this study.

The electrophoretic mobility profile of DNA fragments with 3′-phosphate ends compared with 3′-hydroxyl ends has been previously reported for denaturing polyacrylamide gels [26,41,42]. These studies all reported that 3′-phosphate Maxam–Gilbert sequencing products migrated faster than the corresponding DNA fragments bearing 3′-hydroxyl by less than one nucleotide. This is consistent with the data from our study where the discrepancy in the mobility of these two species was 0.63 nucleotides on average. In addition, these studies also observed that the discrepancy was greater for shorter fragments and progressively reduced for larger ones.

4.3. Bleomycin damage of the 5′-FAM-labelled T7 PCR product

Several differences can be observed when comparing bleomycin damage of the 5′-labelled DNA with that of the 3′-labelled counterpart [13,23]. In contrast to the 3′-labelled DNA, bleomycin damage of the 5′-labelled DNA produced fragments that migrated faster than the DNA fragments produced by the Maxam–Gilbert G + A sequencing by 3–5 nucleotides. This was due to bleomycin cleavage producing 3′-phosphoglycolate termini that migrated anomalously in capillary electrophoresis. We have experimentally verified this result with two other DNA sequences. Previous studies also found that bleomycin damage of the 5′-labelled DNA generated DNA fragments with 3′-phosphoglycolate termini migrating slightly faster than those of the Maxam–Gilbert sequencing size markers on 15–20% denaturing polyacrylamide gels [24,25]. However, this variation was not detected on 8% denaturing polyacrylamide gels

[48,49]. With the polyacrylamide electrophoresis system, this discrepancy in electrophoretic mobility was consistently less than one nucleotide. Therefore, the sites of bleomycin damage could be determined in these studies with 5′-labelled DNA. For example, Georgelin et al. damaged 5′-labelled oligonucleotides containing telomeric sequence with bleomycin and electrophoresed the damaged products on a 15% denaturing polyacrylamide gel [50]. They also found that bleomycin cleaved telomeric sequences mainly at 5′-GT and were able to disregard the discrepancy in electrophoretic mobility between fragments of DNA cleaved by bleomycin and those of the G + A sequencing. However, with the automated capillary sequencer used in this study, the mobility discrepancy was 3–6 nucleotides that made it more difficult to precisely locate the damage sites using 5′-end labelling.

Experiments were performed on 3′-phosphoglycolate termini, by conducting an enzymatic treatment of bleomycin-damaged 5′-labelled DNA. Several enzymes are known to be able to remove the 3′-phosphoglycolate ends including Ape1 and endonuclease IV [28]. These enzymes possess 3′-phosphodiesterase activity which converts the 3′-phosphoglycolate terminus to a 3′-hydroxyl terminus. This treatment is expected to decrease the mobility of the DNA fragment by 3–6 nucleotides. After modification of the bleomycin-damaged DNA fragments with endonuclease IV, the resulting 3′-hydroxyl DNA fragments co-migrated with those derived from the minus sequencing and dideoxy DNA sequencing as expected (Fig. 6). These experiments are consistent with the bleomycin-produced 3′-phosphoglycolate termini having an anomalous mobility on capillary electrophoresis and being converted to 3′-hydroxyl termini by endonuclease IV. Thus these experiments with endonuclease IV affirm the identity of the anomalously migrating bleomycin-cleavage products as 3′-phosphoglycolate termini.

Although DNA fragments bearing 3′-phosphoglycolate have been reported to migrate faster than the corresponding fragments bearing 3′-phosphate produced by Maxam–Gilbert sequencing on the polyacrylamide electrophoresis system [27,28], the difference in electrophoretic mobilities between these two species was considerably higher with the automated capillary sequencer. In previous studies using polyacrylamide electrophoresis, DNA fragments with 3′-phosphoglycolate were found to migrate faster by less than one nucleotide compared with the corresponding fragments bearing 3′-phosphate [27,37,51], while this discrepancy was 3–6 nucleotides in our study using the automated capillary sequencer.

The faster mobility of the 3′-phosphate termini was probably due to the increased net negative charge on the DNA molecule [26]. This will lead to an increase in the charge to mass ratio and therefore an increased mobility towards the positive electrode. However, the greatly increased mobility of the 3′-phosphoglycolate termini is more difficult to rationalise. The 3′-phosphoglycolate will add 2 net negative charges to the DNA fragment compared with an increase of 1 net negative charge for the 3′-phosphate termini. The average change in mobility was 0.63 nucleotides for 3′-phosphate and 4.42 nucleotides for 3′-phosphoglycolate termini; and hence the mobility change cannot be solely due to the increased net negative charge on the 3′-phosphoglycolate termini.

The anomalous migration of 3′-phosphoglycolate termini could be due to a reduced interaction of the terminal nucleotide with the sieving matrix [52,53]. This could lead to reduced hydrogen bonding, hydrophobic or ionic interactions with the sieving matrix. However, it is expected that the strong denaturants, urea and 2-pyrrolidinone, in the electrolyte buffer and high temperature electrophoresis would severely impede such interactions [54].

An electrophoretic mobility change could be caused by an alteration in the secondary structure of the DNA due to intramolecular hairpins and foldbacks [54–56]. The presence of strong denaturants in the electrolyte buffer has been found to eliminate the compressions and reduce secondary structure [8,54,55]. Bowling et al. have postulated that the amino groups on cytosine and adenine are able to hydrogen-bond with the previous nucleotide even in the presence of strong denaturants [54]. They further hypothesised that since this interaction was intramolecular, the effects of the strong denaturants were greatly reduced. Using a similar explanation, the 3′-phosphoglycolate termini could interact intramolecularly with nucleotides in the same DNA molecule by looping and cause a conformational change in the 3′-phosphoglycolate terminated fragments that migrate with a higher mobility on the capillary gels.

5. Conclusions

The electrophoretic mobility of a DNA fragment is an important factor in determining the precise sequence specificity of a DNA damaging agent. The nature of the 3′-temini has a significant effect on the electrophoretic mobility of the DNA fragment, especially 3′-phosphoglycolate termini. Using the automated sequencer coupled with CE-LIF, we have demonstrated that DNA fragments of the same length but differing at the 3′-terminus, possess distinct profiles of electrophoretic mobilities. The electrophoretic mobility of 3′-hydrogen and 3′-hydroxyl termini was similar, while 3′-phosphate termini was faster by 0.63 nucleotides. However, the behaviour of the 3′-phosphoglycolate termini was anomalous and migrated 4.42 nucleotides faster than the 3′-hydroxyl termini. Results obtained from this set of experiments will be helpful in the use of CE-LIF to determine the sequence specificity of DNA-damaging agents and other DNA fragment analysis studies.

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