

Base composition analysis of oligonucleotides containing apurinic sites

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Received June 1, 1993; Revised and Accepted August 11, 1993

ABSTRACT

Upon base composition analysis, oligonucleotides which are labeled at the 3'-terminus with fluorescein or biotin generate an additional, late eluting peak in the HPLC chromatogram. Investigation of this effect revealed the haptens acted as apurinic sites, and phosphodiesterase cleavage of the phosphate bond between the upstream nucleotide and apurinic site is inhibited. Extension of this work with a base-stable apurinic site inserted into all possible junctures of 5'-TGAC-3' tetramers showed this to be a general effect. As a consequence of this work, acid-catalyzed depurination resulting in apurinic sites can be monitored in oligonucleotide synthesis.

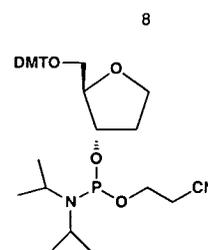
INTRODUCTION

Nucleotide base composition analysis (1), the treatment of DNA with snake venom phosphodiesterase (SVP) and alkaline phosphatase, followed by HPLC analysis for the individual bases, is an important tool for determining the quality of synthetic DNA. One reaction which has a deleterious effect on the viability of DNA is acid-catalyzed depurination. When depurination occurs, an abasic site is formed in the DNA strand. This site is very susceptible to base-induced cleavage, giving a truncated DNA sequence as the product (2). In the course of establishing quality control procedures for covalently fluoresceinated and biotinylated oligonucleotides, we studied the effect of fluorescein and biotin on the phosphodiesterase digestion in base analysis. This work led, in turn, to studies of the effect of a base-stable apurinic site on the SVP digestion. In so doing, we expanded the scope of Weinfeld's observations (3) regarding SVP site specificity.

EXPERIMENTAL

Oligonucleotides were synthesized on an Applied Biosystems M381 DNA Synthesizer using 2-cyanoethylphosphoramidite coupling chemistry. The crude oligonucleotides were deblocked at 55°C in concentrated ammonium hydroxide overnight, then evaporated to dryness. Detritylations of deblocked oligonucleotides were done with 0.1 M AcOH for 1 h at ambient temperature. After adding 25 µL of concentrated ammonium hydroxide to quench the acid, the solvent was evaporated.

Terminal amino groups were emplaced using Aminomodifier II from Clontech Laboratories. Haptentions of amino-terminated oligonucleotides were performed by a literature method (4). The 1',2'-dideoxyribose abasic phosphoramidite **1** was prepared by the method of Eritja (5), as modified by Dervan (6).



Alkaline phosphatase, 4.8 mg/mL in 50% glycerol and phosphodiesterase I (snake venom phosphodiesterase) 1 mg/mL in distilled water, were sourced from Sigma Chemical Co. and stored at -20°C.

HPLC analyses were performed using a Waters Associates system, comprised of a 600E system controller, 745 Data Module, and a 484 UV Detector. The detector was set to 260 nm, with the data module recording at 1.0 AUFS. All other HPLC equipment was obtained from Waters Associates.

Procedure for base composition analysis

To a solution of 0.2 A₂₆₀ units (approx. 7 µg) of single-stranded DNA in 50 µL of 100 mM triethylammonium acetate, pH 7.0, (TEAA) were added 2.8 µL of alkaline phosphatase and 2.4 µL of phosphodiesterase I. After vortexing briefly and centrifuging at 12,560 g for 30 s, the mixture was incubated for 2 h at 50°C. After centrifuging to remove denatured protein, the supernatant was assayed for component nucleosides by HPLC on a 4 µm Nova-Pak cartridge, pressurized to 40 kg/cm² in an RCM 8×10 cartridge holder. The solvents for the gradient analysis were TEAA, solvent A; and acetonitrile, solvent B. The gradient table used was: initial, 3 mL/min, 98% A, 2% B; 5 min, 4 mL/min, 86% A, 14% B; 6 min, 4 mL/min, 86% A, 14% B; 8 min, 4 mL/min, 79% A, 21% B, all linear gradients. A blank run was

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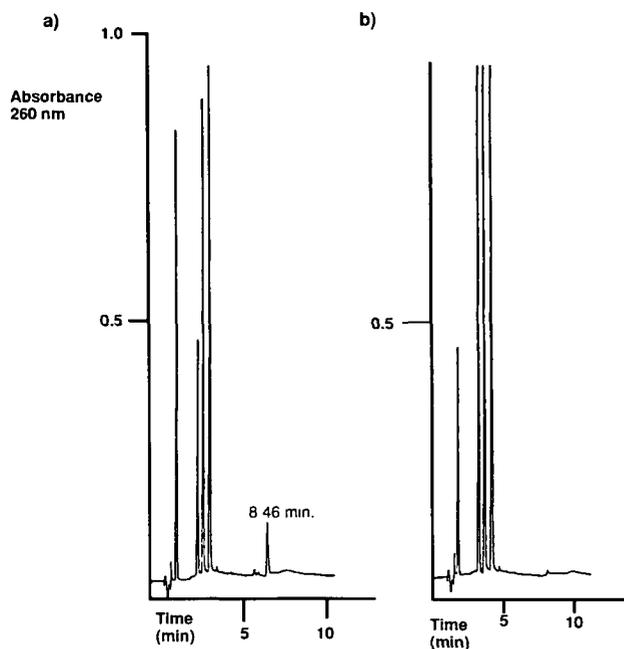


Figure 1: HPLC profile comparison of 24-mer labeled at the 3'-terminus with biotin (a) with a 25-mer labeled at the 5'-terminus with biotin (b). The peak at 8.46 min RT in (a) is due to dA-biotin.

Table 1.

Sequence	Compound #
5'-XRTGAC-3'	2
5'-RTGAC-3'	3
5'-TRGAC-3'	4
5'-TGRAC-3'	5
5'-TGARC-3'	6
5'-TGACR-3'	7

X=Fluorescein isothiocyanate labeled aminomodifier II
R=1',2'-dideoxyribose residue

Table 2.

Compound	Nucleoside-R	Time Difference (min)
4	T-R	1.3
5	G-R	1.3
6	A-R	0.8
7	C-R-X	6.7

X=Fluorescein isothiocyanate labeled aminomodifier II
R=1',2'-dideoxyribose residue

always made before injection of the first sample. While injection of the enzyme digest supernatant entails the potential risk of fouling the HPLC column, this was not observed in practice.

RESULTS AND DISCUSSION

In the course of assaying the integrity of fluorescein and biotin labeled oligonucleotides by base composition analysis, we noted a difference between HPLC chromatograms of SVP digested 5'- and 3'-labeled oligonucleotides. If the oligonucleotides were 5'-labeled, HPLC quantitation of the derived nucleosides afforded

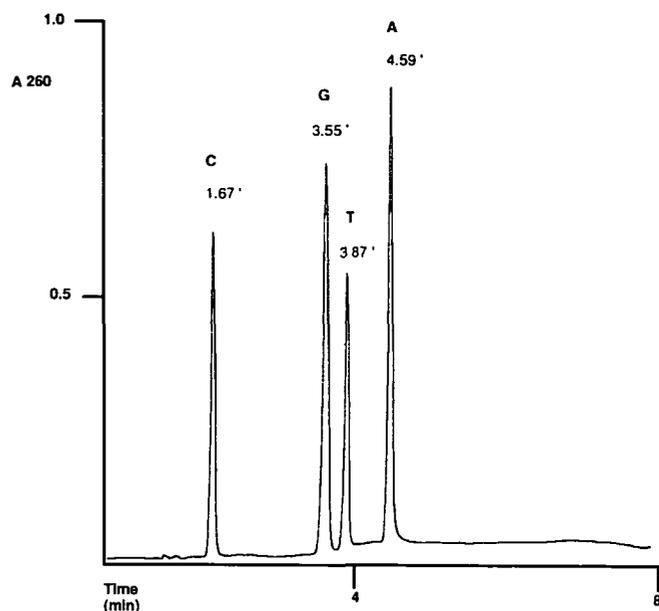


Figure 2. Hydrolysis of RTGAC, 3.

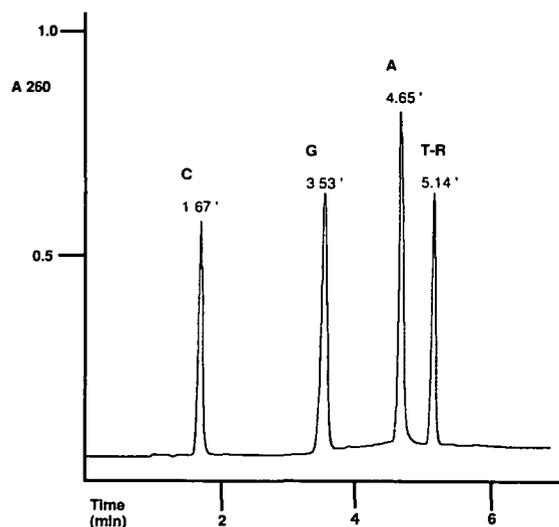


Figure 3. Hydrolysis of 4, TRGAC.

theoretical values within experimental error. If the oligonucleotide was 3'-labeled, an additional, late eluting peak was observed in the chromatogram. Further, analysis of the 4 normal bases agreed with theory only if the base at the 3' end was excluded from the total base calculation. Sample chromatograms are shown in Figure 1a, for a 3'-biotinylated oligonucleotide, and Figure 1b, for a 5'-biotinylated oligonucleotide. We surmised that the phosphodiester bond between 3'-terminal base and the label molecule was not being cleaved by the SVP. The results of Weinfeld (3) with adenosine dimers supported our premise. With 5'-labeled oligonucleotides, the phosphodiester bond between hapten and base was a substrate for SVP. Intrigued by this effect,

we wished to explore its generality. We investigated whether the SVP cleavage pattern was due to positional effects or to the presence of the labeling moieties. To establish the operative effect, we required an apurinic site which was stable to base. Eritja (5) has shown that a 1,2-dideoxyfuranose is stable to high pH. The 2-cyanoethylphosphoramidite **1** was prepared by standard procedures (6,7). We then used phosphoramidite **1** to synthesize a series of 5'-TGAC-3' tetramers which were singly substituted with an apurinic site at all possible junctures. The oligonucleotides were prepared on an automated DNA synthesizer, then purified by reversed-phase HPLC and detritylated. The sequences are summarized in Table 1.

After enzymatic degradations of the oligonucleotides in Table 1, the composition of residual bases was analyzed by reversed-phase HPLC under the conditions cited in the experimental section. If one of the bases from digestion had a longer retention time than the natural nucleoside, we concluded that the digested base was covalently linked to the apurinic site. A similar analysis was used to probe C-4' hydroxylated sites in oxidized DNA (8). Figures 2 and 3 show results from digestions of compounds **3** and **4**. Digestion of oligo **3** gave the normal pattern of nucleosides, indistinguishable from an equimolar mixture of natural DNA nucleosides. Digestion of oligo **4** showed no normal thymine in the sample. Instead, a peak which is most likely 5'-thymine-1,2-dideoxyribose was seen, eluting approximately 1.3 min after the expected thymine retention time. In oligos **4-7**, the HPLC analysis showed the 'loss' of the nucleoside to the 5'-side of the dideoxyribose residue, with the appearance of a new nucleoside peak at longer retention time. In analogy to the thymine case, this peak is most likely 5'-nucleoside-1',2'-dideoxyribose. The relative HPLC retention times of new nucleoside dimers found in enzymatic digestions of **4-7** are listed in Table 2.

From these data, we concluded that the observed SVP cleavage pattern of labeled oligos was derived from the position of the labeling moieties, and not from the labels themselves. On a deeper level, we conclude that SVP requires the presence of a base residue on the 3'-side of a phosphodiester bond in an oligonucleotide for cleavage of that bond to occur. This implies that the enzyme active site will accommodate at least two bases, and that one of the nucleotide bases must be recognized as such by the active site. Finally, the DNA cleavage patterns observed in the hydrolysis of oligomers **2-7** should assist in identification of products from acid-catalyzed depurination of oligonucleotides, since the types of hydrolysis products will be nearly identical for model compounds **2-7** and depurinated DNA.

CONCLUSIONS

Snake venom phosphodiesterase digestion of an oligonucleotide containing an apurinic site to the 3'-side of a viable base gives a dimeric product which resists further hydrolysis by the enzyme. This cleavage pattern is useful in checking the integrity of synthetic oligonucleotides.

ACKNOWLEDGEMENTS

We gratefully acknowledge Clifford Chan for oligonucleotide syntheses and Dr Carolyn Mullen for helpful discussions. We are also grateful to a reviewer for insights regarding possible fouling of the equipment used in this study.

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