THE PRIMARY STRUCTURE OF OLIGONUCLEOTIDES. PARTIAL APURINIZATION AS A METHOD TO DETERMINE THE POSITIONS OF PURINE AND PYRIMIDINE RESIDUES*

E.D. SVERDLOV, G.S. MONASTYRSKAYA, A.V. CHESTUKHIN and E.I. BUDOWSKY
Shemyakin Institute for Chemistry of Natural Products of the Academy of Sciences, Moscow, USSR

Received 10 April 1973

1. Introduction

The absence of specific endonucleases represents an important problem in determination of primary structure of deoxyribo oligonucleotides. As a possible solution of this problem we proposed earlier [1] a novel approach based upon incomplete (random) specific chemical modification followed by determination of position of the modified residues in oligonucleotide. The validity of the approach was illustrated by a method of determination of the positions of cytosine residues in deoxyribo oligonucleotide [1].

Now we propose a method to find the positions of purine and consequently pyrimidine residues in deoxyribo poly- and oligonucleotides.

2. Materials and methods

Alkaline phosphatase of *E. coli* (EC 3.1.3.1) was the product of the special Bureau of Design and Technology of Biologically-Active Substances (Novosibirsk). The stock solution of the enzyme (25 standard units per 1 ml) was in 1 M NaCl – 0.05 M Tris-HCl pH 8.5.

Gurza (*Vipera lebetina*) venom phosphodiesterase was obtained according to Nikolskaya et al. [2]; its stock solution (4 standard units per 1 ml) was in 0.01 M Tris-HCl pH 8.2 – 0.01 $MgCl_2$.

Polynucleotide kinase was obtained according to Richardson [3]. The preparation had a specific activity of 40 000 units per 1 mg of protein and was not contaminated by endo- or exo-nucleases; its stock solution had an activity of 1000 units per 1 ml.

γ-[^{32}P]ATP was obtained by the method of Glinn and Chappel [4]; its specific radioactivity was 3–6 $\times 10^8$ cpm per μmole.

The synthetic deoxyribo oligonucleotide pTpGpTpG has been generously provided by Prof. Z.A. Shabarova and Dr. V. Kagramanov of the Moscow State University.

To introduce the ^{32}P-label into the 5′-position of the oligonucleotide pTpGpTpG, a modification of the method proposed for DNA [5] was employed. 50 μl of 0.2 M Tris-HCl pH 8.0 and 20 μl of *E. coli* alkaline phosphatase was added to 0.5 ml of the oligonucleotide aqueous solution (2 A_{260} units). The mixture was kept for 1 hr at 37°C, and the enzyme inhibited by addition of 60 μl 0.1 M phosphate buffer pH 7.5 This was followed by addition of Tris-HCl pH 7.5, $MgCl_2$ and mercaptoethanol to concentrations 0.05 M, 0.01 M and 0.01 M, respectively. Fifty units of polynucleotide kinase and 80 nmoles of γ-[^{32}P]ATP were added, and the mixture was kept at 37°C for 1 hr. The proteins were removed by extraction with phenol (6 × 1 ml) saturated with 0.1 M phosphate buffer pH 7.5. Phenol was removed by extraction with ether, and 70 μl of the mixture was subjected to gel-filtration on a Sephadex G-10 column (0.12 × 20 cm) to remove the excess of γ-[^{32}P]ATP. The oligonucleotide which came off the column as the first peak was purified by micro-column chromatography [1, 6, 7] on a standard column (10 cm long, 50 μl) with DEAE-cellulose (TLC, Serva); the elution was performed by a linear gradient (600 μl) of NaCl (0 → 0.3 M) in 7 M

* Part II, part I, see [1].

urea – 0.02 M Tris-HCl pH 7.5*. For final purification, the oligonucleotide was rechromatographed under the same conditions; the yield of the finally purified oligonucleotide was 5×10^{-2} A_{260} units (1000 cpm).

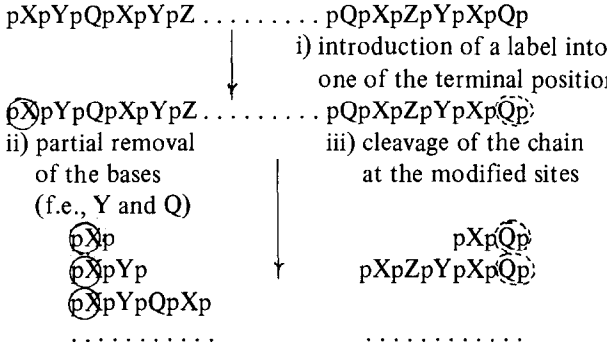
2.1. *Partial apurinization*

2 μl (0.46 A_{260} units) of Burton-Petersen [8] DNA digest and 2 μl (5×10^{-3} A_{260} units of each) pT and pC mixture were added to 2.5×10^{-2} A_{260} units (500 cpm) of ^{32}pTpGpTpG. The mixture was evaporated to dryness in a micro-tube by air stream, 50 μl of 66% formic acid – 2% diphenylamine added, and apurinization was run at 37°C for 45 min (approx. half-life time of the reaction). The cooled mixture was applied under drying with a strong stream of cold air onto a strip of sterile filter paper as a spot of 3–4 mm diameter. Diphenylamine and formic acid were removed by ascending elution with ether (the lower end of the strip was immersed into a tube with ether, and the upper end was left in the air). The paper was dried, the spot cut out and the oligonucleotides extracted with 300 μl of 2×10^{-3} M Tris-HCl pH 7 in 7 M urea during 12 hr at 50°C; 30 μl of 0.2 M Tris-HCl pH 8.2 – 10^{-2} M $MgCl_2$ and 10 μl of phosphodiesterase stock solution was added to the mixture, and it was kept for 1 hr at 37°C and for 1 hr at 100°C. The filter was removed and washed with 250 μl of water. The mixture was subjected to micro-column chromatography on a standard column with 600 μl of NaCl linear gradient (0 → 0.25 M) in 7 M urea – 0.05 M sodium acetate pH 5.5. Fractions were collected corresponding to peaks and to intervals between them and their radioactivity was counted using Bray's scintillating liquor.

3. Results and discussion

In our previous paper [1] we reported a method for determination of the positions of the given type(s) of nucleoside residues in oligonucleotides. The method is based upon: i) incomplete specific chemical modification restricting the action of nucleases; ii) separation of the mixture of monomodified oligonucleotides from other compounds; iii) action of phosphodiesterase on this mixture; iv) determination of the chain length of the resulting oligonucleotides, terminated by a modified residue. The length of the oligonucleotides obtained after phosphodiesterase action corresponded to the distances between one or the other terminus of the starting oligonucleotide (depending on the type of the phosphodiesterase) and modifies residues, i.e. residues of the given type(s).

This approach – incomplete specific chemical modification – could be employed also in another manner, illustrated in the following scheme:



The number of nucleotide residues in the labelled products of the last stage correspond to the distances between the labelled end of the starting poly- or oligonucleotide and the residues of the given type(s).

In the present study we have confirmed the validity of the new approach using partial apurinization, which allows one to determine the positions of purine (and, consequently, pyrimidine) residues in deoxyribo poly- or oligonucleotides. The model compound was the synthetic deoxyribo tetranucleotide – pTpGpTpG with ^{32}P-labelled 5-terminal phosphate; the label was introduced with polynucleotide kinase after phosphatase treatment.

The partial apurinization and simultaneous cleavage of the adjacent phosphodiester bonds resulting in an oligonucleotides mixture of the general fomula $p(Np)_n$, was performed by means of Burton–Petersen reagent [8] (diphenylamine–formic acid). It followed from the data of Burton and Petersen [8] that the rate-limiting stage of the process was apurinization, and, hence, the amounts of apurinized, but uncleaved oligonucleotides should be insignificant. In contrast to the oligonucleotides with 3′-phosphate grouping, formed in this procedure, the starting oligonucleotide could be easily removed by the action of the snake venom phospho-

* The same standard column and techniques were employed in the present studies in all the chromatographic runs.

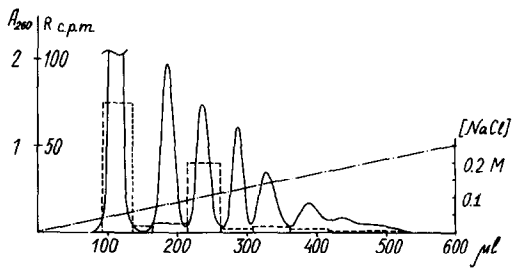


Fig. 1. Determination of the chain length of labelled compounds, formed by partial apurinization and splitting of ^{32}pTpGpTpG (see Materials and methods for details). (——) Optical density at 260 nm; (- - - -) radioactivity; (·—·—·) concentration of NaCl.

diesterase (cf. [9]). For this reason the reaction mixture after incubation with diphenylamine–formic acid was extracted with ether and subjected to snake venom phosphodiesterase treatment under the conditions leading to complete digestion of the starting oligonucleotide.

The micro-column chromatography of the digestion on DEAE-cellulose with a non-labelled carrier (Burton–Petersen digest of DNA) shows two peaks of radioactivity overlapping with the A_{260} peaks of mononucleoside diphosphate and trinucleoside tetraphosphate (see fig. 1). This pattern corresponded to the distribution of the purine residues in the oligonucleotide used – pTpGpTpG and demonstrated the validity of the approach.

The method proposed is very simple, it needs minute amounts of material and can be employed for estimation of the distribution of the purine (and, consequently, pyrimidine) residues in deoxyribo oligonucleotides or in the end sequences of deoxypolynucleotides.

Acknowledgements

The authors are greatly indebted to Prof. Z.A. Shabarova and Dr. V. Kagramanov for their gift of tetranucleotide.

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