The Fractionation of Adenosine-Rich Oligoribonucleotides on Polyethyleneimine-Cellulose

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After combined pancreatic and T_1 ribonuclease treatment of RNA, a characteristic series of products of the type A_nN are obtained, where N can be any of the four ribonucleosides. Depending on whether phosphatase treatment is used before the addition of labelled phosphate at the 5'-terminus with bacteriophage phosphokinase, the labelled oligonucleotides obtained may or may not possess a phosphate group at the 3'- as well as the 5'-end. The behaviour of these characteristic products after electrophoresis on cellulose acetate strips followed by chromatography on polyethyleneimine-cellulose thin-layer plates was examined.

Several reports have appeared on the use of polyethyleneimine-cellulose for the one- or two-dimensional separation of both deoxyribo- and ribonucleotides (Randerath & Randerath, 1967; Southern & Mitchell, 1971; Griffin, 1971). The compact spots obtained made the method particularly suitable for the separation of very small amounts of oligonucleotides. In the course of sequence studies of mouse globin mRNA, it was necessary to characterize the behaviour of the oligonucleotide products of a digest with pancreatic plus T_1 RNAases.*

Pancreatic RNAase hydrolyses internucleotide bonds in RNA specifically after cytidine and uridine residues, giving first a 2':3'-cyclic phosphate and then in turn hydrolysing this to the 3'-phosphate. Poly(A) sequences are also hydrolysed slowly to give oligo(A) fragments by pancreatic RNAase, or by a minor contaminating enzyme activity, particularly in solutions of low ionic strength (Beers, 1960). T_1 RNAase hydrolyses internucleotide bonds specifically after guanosine residues, giving oligonucleotides terminating in the 3'-phosphate. Bacterial alkaline phosphatase will hydrolyse 3'-phosphate groups (as well as 5'-phosphate groups), but will not attack 2':3'-cyclic phosphates or phosphodiester bonds. Bacteriophage phosphokinase transfers a phosphate group from the γ -position of ATP to the 5'-end of an oligonucleotide or a mononucleotide, but not to a mononucleoside. Therefore, depending on whether the terminal phosphate is cyclic or not, and whether phosphatase is used, series of oligonucleotides of the form $(pA)_n pN-OH$ and $(pA)_n pNp$ will be generated after treatment of RNA with T_1 plus pancreatic RNAases, where N can be any of the four ribonucleosides.

* Abbreviation: RNAase, ribonuclease.

Materials and Methods

Pancreatic RNAase A, poly(A,G), poly(A,C) and poly(A,U) were purchased from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; poly(A), poly(U) and poly(C) were from Miles Laboratories, Stoke Poges, Slough, U.K.; RNAase T_1 was from Sankyo Co., Tokyo, Japan; and bacterial alkaline phosphatase was from Worthington Biochemical Co., Freehold, N.J., U.S.A. Polynucleotide kinase, which was substantially RNAase-free, was a kind gift from Dr. K. Murray, Department of Molecular Biology, University of Edinburgh, U.K., and was prepared as described by Richardson (1965). [γ - ^{32}P]ATP was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., at a specific radioactivity of 15 Ci/mmol. Polyethyleneimine-cellulose plates (20 cm $\times$ 40 cm) were obtained from Macherey, Nagel and Co., Düren, Germany, and Cellogel strips were from Reeve Angel Scientific Co. Ltd., Maidstone, Kent, U.K.

Digestion of RNA with RNAases

Polyribonucleotide (5–10 μ g) was used for each digestion, which was carried out in 10 μ l of 0.01 M-Tris-HCl, pH 7.4, containing both T_1 and pancreatic RNAases each at 0.05 mg/ml. The substrate/enzyme ratio was thus between 10:1 and 20:1 (w/w). Incubation was for 45 min at 37°C.

When subsequent treatment with phosphatase was not required, the reaction was terminated by the addition of an equal volume of water-saturated phenol-chloroform (1:1, v/v). After two extractions the aqueous phase was dried down on to a polythene sheet under vacuum. If phosphatase treatment was required, sodium dodecyl sulphate was added to

10mg/ml and bacterial alkaline phosphatase to 0.1mg/ml and incubation was carried out for a further 30min at 37°C. The reaction was terminated by the addition of KCl solution to 0.035M, followed by an equal volume of water-saturated phenol-chloroform (1:1, v/v). The RNA was extracted twice and dried down.

Labelling of oligonucleotides with phosphokinase

The digested RNA was taken up in 1 μ l of 0.1M-Tris-HCl (pH 7.5)–11 mM-2-mercaptoethanol–11 mM-MgCl₂–0.27 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ containing approx. 1 unit of polynucleotide kinase (1 unit is the amount of enzyme catalysing the formation of 1 nmol of acid-insoluble ^{32}P in 30min at 37°C). Incubation was carried out at 37°C for 45min (Szekely & Sanger, 1969). The mixture was dried down under vacuum, and the ^{32}P -labelled oligonucleotides were taken up in 0.7 μ l of dye mixture (Brownlee, 1972) and separated by using techniques based on those described by Southern & Mitchell (1971).

Separation methods

The oligonucleotide mixture was applied to 47 cm-long Cellogel strips equilibrated with 7M-urea–1 mM-EDTA–5% (v/v) acetic acid, adjusted to pH 4.2 with pyridine. Electrophoresis was carried out at 3 kV until the pink and blue marker spots had separated by approx. 11 cm, usually for 45–50 min. The position of the oligonucleotides was determined with a hand monitor (from just past the pink marker dye to within 2–3 cm of the origin) and they were transferred to a pre-wetted polyethyleneimine-cellulose plate by using filter-paper wicks and a glass rod, a technique developed by Dr. E. M. Southern

(personal communication), which gives a very thin streak and quantitative transfer. Chromatography in the second dimension was with 1.5M-formate buffer adjusted to pH 3.5 with pyridine. After the solvent front had risen overnight to within a few cm of the top of the 40 cm thin-layer plate, the chromatograms were dried, marked with radioactive ink and radioautographed by using Kodirex X-ray film.

Results

Poly(A,C), poly(A,G) and poly(A,U), after treatment with pancreatic and T₁ RNAses give mixtures of oligo(A) fragments plus fragments with various numbers of adenosine residues terminating in cytidine, guanosine or uridine. All oligonucleotides detected are labelled with $[\text{P}^{32}]\text{phosphate}$ on the 5'-terminus. If phosphatase is used (Figs. 1a, 2a, 3a) the oligonucleotides have no phosphate on the 3'-terminus; if phosphatase is omitted there are phosphate groups on both 3'- and 5'-ends, and mononucleotides are also labelled (Figs. 1b, 2b, 3b). With poly(A) (Fig. 4), the isopliths obtained are similar because the cyclic phosphate generated by the action of pancreatic RNAase (or some contaminant of pancreatic RNAase) is not hydrolysed to the 3'-phosphate and is therefore not susceptible to the action of bacterial alkaline phosphatase (Markham, 1957). The small amount of (pA)_npA-OH seen presumably represents the 3'-terminal fragments.

The mixture of poly(A), poly(G), poly(C) and poly(U), phosphorylated without phosphatase treatment after enzymic digestion is shown in Fig. 5. Only the oligo(A) fragments and pCp, pGp and pUp are seen. Fig. 6 shows the 'fingerprint' obtained after

Fig. 1. Two-dimensional 'fingerprint' of pancreatic plus T₁ RNAase digest of poly(A,C) followed by phosphokinase labelling. Experimental details are given in the Materials and Methods section. (a) With phosphatase treatment; (b) without phosphatase treatment. The radioautograph from which Fig. 1(b) is obtained is shown in Plate 1(a). PEI, polyethyleneimine.

(a)

(b)

(c)

(d)

EXPLANATION OF PLATE I

Radioautographs of two-dimensional 'fingerprints' of pancreatic plus T_1 RNAase digests

(a) poly(A,C) without phosphatase treatment (oligonucleotides identified in Fig. 1b); (b) poly(A,U) without phosphatase treatment (oligonucleotides identified in Fig. 2b); (c) poly(A,G) without phosphatase treatment (oligonucleotides identified in Fig. 3b); (d) poly(A) without phosphatase treatment (oligonucleotides identified in Fig. 4b).

Fig. 2. Two-dimensional 'fingerprint' of pancreatic plus T_1 RNAase digest of poly(A,U)

Experimental details are given in the Materials and Methods section. (a) With phosphatase treatment; (b) without phosphatase treatment. The radioautograph from which Fig. 2(b) is obtained is shown in Plate 1(b). PEI, polyethyleneimine.

Fig. 3. Two-dimensional 'fingerprint' of pancreatic plus T_1 RNAase digest of poly(A,G)

Experimental details are given in the Materials and Methods section. (a) With phosphatase treatment; (b) without phosphatase treatment. The radioautograph from which Fig. 3(b) is obtained is shown in Plate 1(c). PEI, polyethyleneimine.

Fig. 4. Two-dimensional 'fingerprint' of pancreatic plus T_1 RNAase digest of poly(A)

Experimental details are given in the Materials and Methods section. (a) With phosphatase treatment; (b) without phosphatase treatment. The radioautograph from which Fig. 4(b) is obtained is shown in Plate 1(d). PEI, polyethyleneimine.

Fig. 5. Two-dimensional 'fingerprint' of pancreatic plus T_1 RNAase digest of a mixture of poly(A), poly(C), poly(G) and poly(U) without phosphatase treatment

Experimental details are given in the Materials and Methods section. PEI, polyethyleneimine.

Fig. 6. Two-dimensional 'fingerprint' of pancreatic plus T_1 RNAase digest of a mixture of poly(A,C), poly(A,U), poly(A,G) and poly(A) without phosphatase treatment

Experimental details are given in the Materials and Methods section. PEI, polyethyleneimine.

enzymic treatment and kinase labelling (but without phosphatase treatment) of a mixture of poly(A,C), poly(A,U), poly(A,G) and poly(A). The characteristic isopliths derived from poly(A) are clearly distinguishable from those terminating in cytidine, guanosine or uridine.

Discussion

Comparison of the 'fingerprints' of the oligonucleotides with and without phosphatase treatment indicates that the presence of a phosphate group on the 3'-terminus increases the mobility of the oligonucleotide in the first dimension, and (with the exception of the isopliths terminating in adenosine) decreases the mobility in the second dimension. With the exception of some overlap between the isopliths $(pA)_n pGp$ and $(pA)_n pUp$, each of the oligonucleotides occupies a unique and identifiable position in the 'fingerprint'. It is apparent that the enzyme activity which hydrolyses poly(A) to oligo(A) does not break the cyclic phosphate into a phosphatase-susceptible 3'-linkage. It is also apparent that commercial poly(A) contains very small amounts of contaminating cytidine (Fig. 4b). Because mononucleotides as well as oligonucleotides are phosphorylated if phosphatase treatment is omitted, this experimental protocol has advantages if quantitative measurement is attempted.

These techniques have been applied by us to the study of the poly(A) region of mouse globin mRNA (Mansbridge *et al.*, 1973). Others have used a mixture of T_1 plus pancreatic RNAases to analyse the methylated base sequences in rRNA (Maden & Forbes, 1972; Maden *et al.*, 1972) by using con-

ventional 'fingerprinting' techniques (Brownlee, 1972). Similar digests of Rous sarcoma virus have been separated by homochromatography (Horst *et al.*, 1972).

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