# TRITIUM SEQUENCE ANALYSIS OF POLYRIBONUCLEOTIDES FOLLOWING PERIODATE-PHOSPHOMONOESTERASE DEGRADATION – CHARACTERIZATION OF OLIGONUCLEOTIDE-3′ DIALDEHYDE INTERMEDIATES*

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Received 2 December 1973

## 1. Introduction

Incubation of polyribonucleotides with periodate and *E. coli* alkaline phosphomonoesterase results in a directional (3′ → 5′) degradation process [1] which leads to the formation of two groups of primary reaction products: 1) nucleoside methylene dialdehydes [1–3] and 2) oligonucleotide-3′ dialdehydes [1]. Consequently, there are two alternatives for analyzing this reaction, one of which makes use of the reduction of nucleoside methylene dialdehydes with $^3$H-labelled borohydride as described previously [1–3]. We have also investigated the second possibility, i.e. the characterization of the oligonucleotide-3′ dialdehyde intermediates and have made the following pertinent observations:

(1) The reaction leads to the formation of a mixture of oligonucleotide-3′ dialdehydes of different chain lengths.

(2) $^3$H-labelled oligonucleotide-3′ dialcohols are obtained by treating the aldehydes with borotritide.

(3) These oligonucleotide dialcohols are readily separated according to chain length by anion-exchange TLC on PEI-cellulose.

(4) $^3$H-labelled nucleoside trialcohols are released from the 3′-termini of the dialcohols by treatment with $T_2$-RNAase.

(5) This enzymatic digestion may be carried out in situ on PEI-cellulose thin layers.

(6) Following $T_2$-RNAase treatment, the radioactive trialcohols may be resolved after direct contact transfer from PEI-cellulose to silica gel thin layers and assayed by scintillation counting.

(7) This scheme provides a novel, highly sensitive means for deducing, by chemical 'postlabelling', the sequence of nonradioactive oligoribonucleotides.

Fig. 1 illustrates the method for the pentanucleotide CpCpCpApGp (=CCCAG).

## 2. Experimental

The reaction mixtures contained: oligonucleotide ($10^{-5}$ M), sodium borate ($3 \times 10^{-2}$ M, the added buffer being 0.1 M $Na_2B_4O_7$–HCl, pH 8.0 at 23°C), *E. coli* alkaline phosphomonoesterase (ribonuclease-free, Worthington code BAPF; 0.06 μg/μl), and $NaIO_4$ ($2 \times 10^{-4}$ M). Incubation was at 50°C in the dark. Aliquots were withdrawn during the reaction and kept at −72°C until borotritide treatment. The reduction was initiated by adding [$^3$H]$KBH_4$ (6.1 Ci/

* This is part XVI of a aeries entitled 'Analysis of Nucleic Acid Derivatives at the Subnanomole Level'. For part XV, see the preceding paper [3].

*Unusual abbreviations*: TLC, thin-layer chromatography; PEI-cellulose, a cellulose anion-exchanger obtained by treating unmodified cellulose with polyethyleneimine (see ref. [5]); N*, a dialdehyde derivative of a nucleoside or nucleotide; N′, a borohydride reduction product of a nucleoside (or nucleotide) dialdehyde.

** Faculty Reserach Awardee of the American Cancer Society.

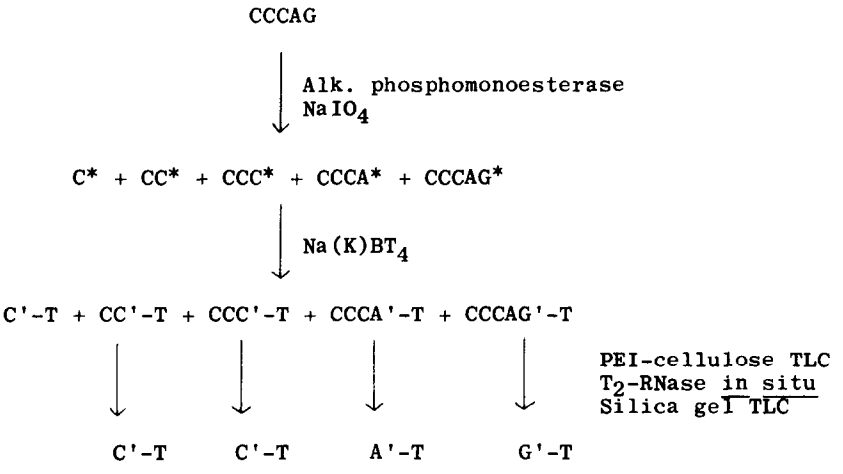


Fig. 1. Tritium sequencing of a pentanucleotide.

mmole, 5-fold molar excess over $NaIO_4$) to each aliquot. After 2 hr at room temperature, acetic acid (final concentration 0.3 M) was added and the solution evaporated. The residue was taken up in water to make the volume of the final labelled solution the same as before addition of borotritide.

2–4 μl of each labelled solution was applied to a PEI-cellulose thin layer, which was prepared as described [4], except that a neutralized (pH 6) 0.5% aqueous solution of PEI-1000, a high-molecular weight polyethyleneimine (Dow Chemical Co., Freeport, Texas) was used. As a marker, an aqueous solution of CpC (5 nmoles) was also applied to the chromatogram. To adjust the pH of the layer the sheet was soaked in methanol/conc. ammonia (1000:1; 500–1000 ml depending on its size). To remove non-phosphorylated nucleotide derivatives, the sheet was developed with distilled water to 5 cm on a Whatman 1 wick fastened with staples 16–17 cm above the origin. After thorough drying in a stream of air, the nucleotides were separated by stepwise development [5] as follows: water to the origin; 0.10 M LiCl to 5 cm above the origin; 0.25 M LiCl to 12 cm; 0.60 M LiCl to about 3 cm on the wick (no intermediate drying). Chromatography was terminated when the CpC marker reached a line about 2.5 cm below the wick. After drying, tritiated compounds were rendered visible by low-temperature fluorography following treatment of the layer with PPO [6] and their location was marked on the layer [7].

To remove PPO and LiCl the sheet was soaked for 5–10 min in methanol and the oligonucleotides were digested in situ with $T_2$-RNAase (1 mg/ml; Sigma R 3751) for 1–2 hr at room temperature: 2–4 μl of enzyme solution was applied to the center of the spot, which was then immediately covered with a piece of Parafilm. After drying, 2 μl of a mixture of nonradioactive nucleoside trialcohols [7], 2–3 mM with respect to each component, was applied at the site of enzyme application. After evaporation, the treated area was cut out and placed in contact with a silica gel layer (Eastman 13181, with indicator). The PEI-cellulose cutout-silica gel assembly was sandwiched between small, but rather strong ('button-type') magnets [5]. Subsequent ascending chromatography quantitatively transfers nucleoside trialcohols from the PEI-cellulose cutout to the silica gel layer. TLC was carried out as detailed elsewhere [1] except that the solvents used were (A) acetonitrile/t-amyl alcohol/conc. ammonia (2:1:1) for the first dimension, and (B) t-amyl alcohol/methyl ethyl ketone/water (3:6:1.2) for the second dimension. This combination results in an excellent separation of the four major trialcohols, similar to one previously published (fig. 3 of ref. [7]).

Compounds were located in UV-light, cut out and assayed by scintillation counting [7].

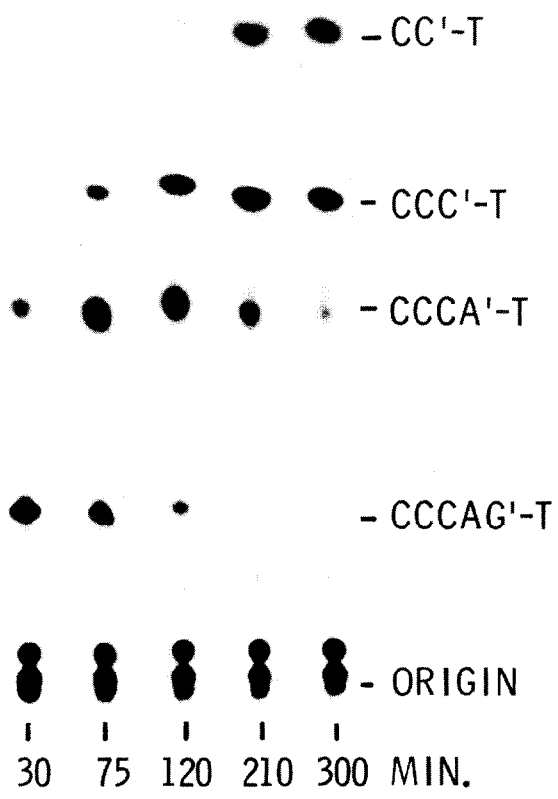


Fig. 2. PEI-cellulose thin-layer chromatogram obtained following periodate-phosphatase degradation of CpCpCpApGp and tritium postlabelling. Film detection by fluorography. For conditions, consult text. The radioactivity of the origin areas and of minor components are due to impurities in the commercial $KBT_4$ preparation and are clearly distinguishable from the radioactive nucleotides.

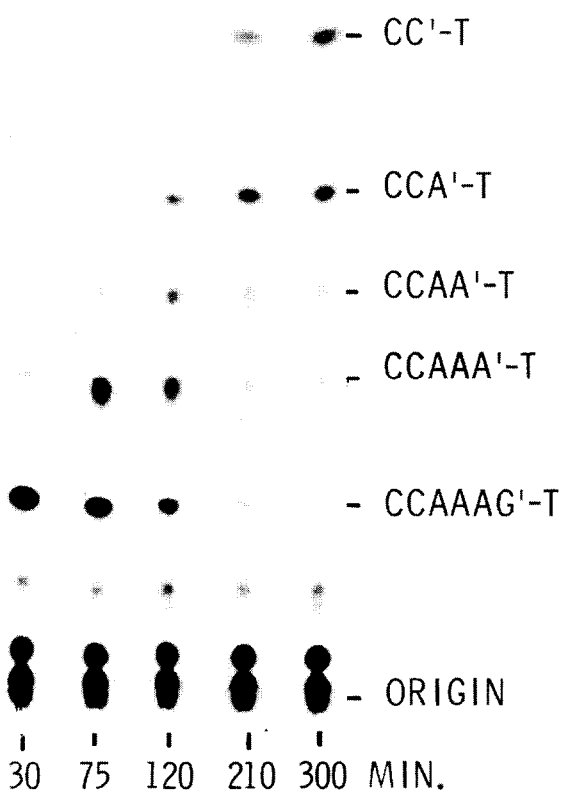


Fig. 3. PEI-cellulose thin-layer chromatogram obtained from CpCpApApApGp. For conditions, consult legend of fig. 2 and text.

## 3. Results and discussion

Results obtained by applying this procedure to the pentanucleotic CpCpCpApGp and the hexanucleotide CpCpApApApGp are shown in figs. 2 and 3 and tables 1 and 2. The data present evidence for the presence of oligonucleotide-3′ dialdehydes in the incubation mixtures. Upon borotritide treatment, these compounds are converted to terminally $^3$H-labelled oligonucleotide-3′ dialcohols cf. fig. 1. $T_2$ RNAase treatment releases the termini as $^3$H-labelled trialcohols. It will be noted (figs. 2 and 3) that during the course of the reaction the larger oligonucleotides disappear as a result of their conversion to pieces of shorter chain lengths. As expected on the basis of the reaction mechanism [1], terminal labelling of the oligonucleotide initially present in the incubation mixture precedes labelling of nucleotides subsequently generated by β-elimination, dephosphorylation, and oxidation. The largest labelled oligonucleotide thus provides a conspicuous marker on the films (figs. 2 and 3).

For separating reaction mixtures derived from large oligonucleotides on PEI-cellulose, solvents containing 8.5 M urea are superior to simple LiCl solvents (unpublished), but the high resolving power of PEI-cellulose for oligonucleotides (8,9) makes the use of urea systems unnecessary in many instances.

Tables 1 and 2 demonstrate that, for deducing the sequence, at least up to a chain length of 6, the ap-

Table 1.
Radioactivity (cpm) of 3′-termini released by in situ $T_2$-RNAase treatment on PEI-cellulose of [$^3$H] oligonucleotide-3′ dialcohol spots following periodate-phosphatase degradation of CpCpCpApGp.

| ($^3$H)Trialcohol* | Reaction time (min) 75 | 120 | 210 | Sequence deduced |
|---|---|---|---|---|
| $A'_5$ | 36 | 28 | 24 | |
| $C'_5$ | 42 | 28 | 12 | . . . pG (p) |
| $U'_5$ | 33 | 32 | 19 | |
| $G'_5$ | 2990 | 1166 | 269 | |
| $A'_4$ | 3122 | 1999 | 1242 | |
| $C'_4$ | 41 | 48 | 45 | |
| $U'_4$ | 25 | 13 | 21 | . . . pApG (p) |
| $G'_4$ | 54 | 34 | 19 | |
| $A'_3$ | 18 | 16 | 25 | |
| $C'_3$ | 1286 | 2988 | 4125 | |
| $U'_3$ | 18 | 17 | 26 | . . . pCpApG (p) |
| $G'_3$ | 19 | 15 | 17 | |
| $A'_2$ | 30 | 26 | 10 | |
| $C'_2$ | 94 | 458 | 2622 | |
| $U'_2$ | 18 | 14 | 19 | . . . pCpCpApG (p) |
| $G'_2$ | 10 | 14 | 19 | |

* The subindex indicates the chain length of the oligonucleotide.

Table 2
Radioactivity (cpm) of 3′-termini released by $T_2$-RNAase treatment of [$^3$H] oligonucleotide-3′dialcohol spots following periodate-phosphatase degradation of CpCpApApApGp.

| ($^3$H)Trialcohol* | Reaction time (min) 75 | 120 | 210 | Sequence deduced |
|---|---|---|---|---|
| $A'_6$ | 22 | 24 | 32 | |
| $C'_6$ | 8 | 22 | 17 | |
| $U'_6$ | 9 | 23 | 15 | . . . pG (p) |
| $G'_6$ | 4352 | 1439 | 317 | |
| $A'_5$ | 1518 | 1216 | 429 | |
| $C'_5$ | 11 | 17 | 23 | |
| $U'_5$ | 23 | 12 | 17 | . . . pApG (p) |
| $G'_5$ | 28 | 21 | 17 | |
| $A'_4$ | 579 | 895 | 627 | |
| $C'_4$ | 20 | 22 | 30 | |
| $U'_4$ | 20 | 14 | 11 | . . . pApApG (p) |
| $G'_4$ | 26 | 21 | 19 | |
| $A'_3$ | 207 | 546 | 1452 | |
| $C'_3$ | 16 | 9 | 18 | |
| $U'_3$ | 7 | 17 | 14 | . . . pApApApG (p) |
| $G'_3$ | 12 | 13 | 24 | |
| $A'_2$ | 12 | 18 | 21 | |
| $C'_2$ | 30 | 154 | 522 | |
| $U'_2$ | 13 | 19 | 13 | . . . pCpApApApG (p) |
| $G'_2$ | 19 | 10 | 22 | |

* The subindex indicates the chain length of the oligonucleotide.

plication of this procedure requires the analysis of only 2–3 properly timed aliquots. This appears to be a distinct advantage when compared with analyzing the process at the level of the methylene dialdehydes [1–3] where a substantially greater number of aliquots has to be assayed to deduce the sequence. The 5′-terminus has to be determined separately, see the preceding paper [3].

On the basis of the results reported in this and the preceding paper it appears that periodate-phosphomonoesterase degradation of polyribonucleotides combined with tritium postlabelling will make possible sequencing small amounts of nonradioactive RNA with similar precision and sensitivity as is now possible by methods based on biological prelabelling [10]. It may be hoped that the development and application of these methods will be significant steps towards an understanding on nucleic acid directed human diseases at the molecular level.

## Acknowledgements

We thank Drs. S.H. Chang, S. Nishimura, and U.L. RajBhandary for generously providing samples of oligonucleotides of known sequence.

This work was supported by USPHS Grants CA-13591 and CA-10893-P8 and American Cancer Society Grant NP-37 and Award PRA-108.

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