TRITIUM SEQUENCE ANALYSIS OF POLYRIBONUCLEOTIDES FOLLOWING PERIODATE-PHOSPHOMONOESTERASE DEGRADATION – ANALYSIS OF NUCLEOSIDE METHYLENE DIALDEHYDES DERIVED FROM TETRA-, PENTA-, AND HEXANUCLEOTIDES*

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1. Introduction

At the present time, the most sensitive and precise sequencing methods for RNA depend on biological prelabelling of the RNA with [^{32}P]phosphate [1]. Although this methodology has been highly successful for sequencing viral and bacterial RNAs, characterization of the structure of many RNAs in human and other mammalian tissues, for reasons discussed elsewhere [2], requires the development of novel methods that are independent of biological labelling. For the past several years, a major objective in our laboratory has been the development of sensitive methods for structural characterization of nonradioactive nucleic acids. A recently discovered degradation process of polyribonucleotides [3] appears to be well adapted to this purpose since the products formed during this reaction are dialdehyde derivatives of nucleosides and oligonucleotides, which are amenable to assay in a highly sensitive manner by reduction with ^{3}H-labelled borohydride. In this connection, it is important that the degradation process itself takes place at extremely low (10^{-7} to 10^{-5} M) oligonucleotide concentration, as demonstrated for trinucleotides [4].

We have now extended the scope of this method to longer oligomers with chain lengths of up to 10 nucleotides. This communication details results obtained with tetra-, penta-, and hexanucleotides. The time course of the reaction was analyzed by borotritide reduction of nucleoside methylene dialdehydes released during incubation with phosphatase and periodate from the polynucleotide chain [3,4]. The following observations were made:

(1) Complete degradation of oligonucleotides up to decamers (and probably larger) takes place.

(2) Tritium postlabelling of nucleoside methylene dialdehydes readily establishes the sequence of oligomers of chain lengths up to six nucleotides.

(3) However, it appears that in many cases, especially for larger oligonucleotides, tritium derivative analysis of oligonucleotide-3′ dialdehydes, as detailed in a companion paper [5], is superior to assaying the methylene derivatives.

(4) Since nucleoside methylene dialdehydes were found to undergo slow hydration during incubation at slightly alkaline pH to give nucleoside dialdehydes the identification of the 5′-terminus of large oligonucleotides may require alternative procedures such as venom phosphodiesterase digestion followed by tritium labelling.

* This is part XV of a series entitled 'Analysis of Nucleic Acid Derivatives at the Subnanomole Level'. Part XIV is 'Base Analysis of Ribopolynucleotides by Chemical Tritium Labelling: An Improved Mapping Procedure for Nucleoside Trialcohols', by K. Randerath, E. Randerath, L.S.Y. Chia, and B.J. Nowak, Anal. Biochem., in press (1973).
Unusual abbreviations: TLC, thin-layer chromatography; N′ (A′, C′, U′, G′), a nucleoside trialcohol; N″ (A″, C″, U″, G″), a nucleoside methylene dialcohol.

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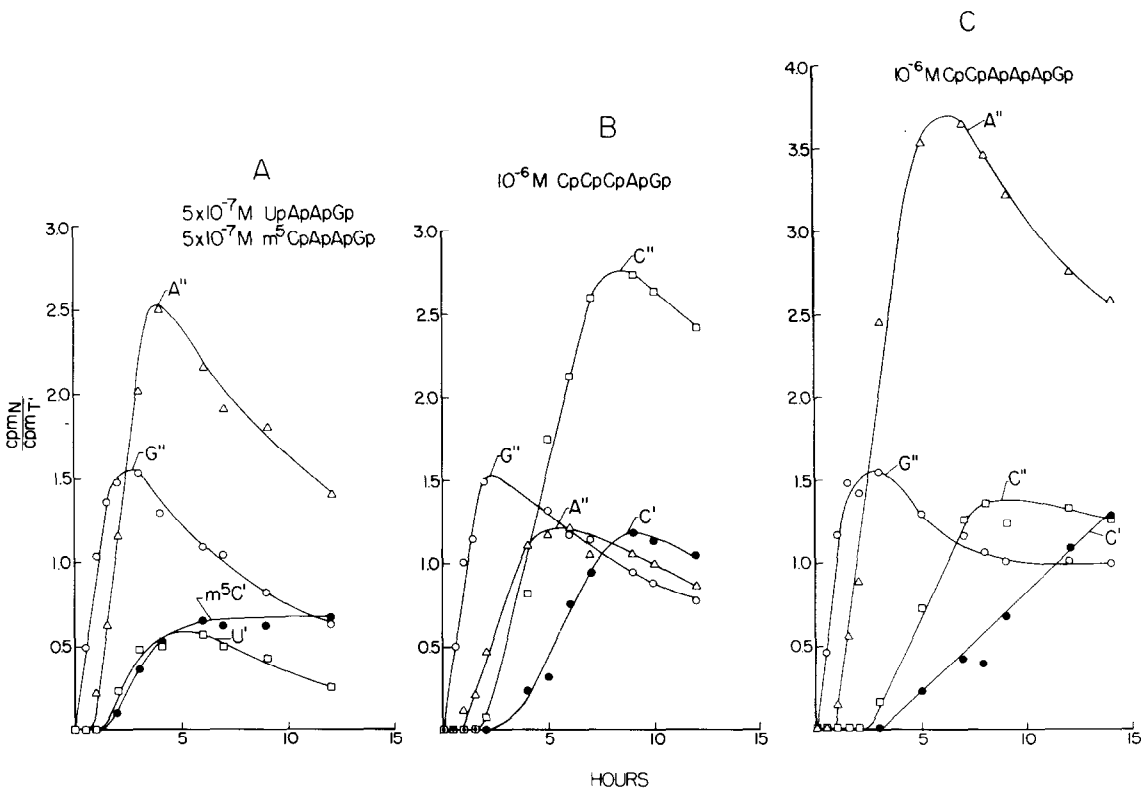

Fig. 1. Degradation of various oligonucleotides by periodate-phosphomonoesterase treatment. Assay of nonphosphorylated dialdehyde derivatives by reduction with [^{3}H]KBH_4,TLC, and scintillation counting. For conditions, consult text.

2. Experimental

Reaction conditions were similar to those previously reported [4]. The reaction mixtures contained: oligonucleotide (10^{-6} M), sodium borate (3×10^{-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; 0.007 μg/μl), and $NaIO_4$ (1.8×10^{-5} 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 (2 Ci/mmole, a 20-fold molar excess over $NaIO_4$) to each aliquot. After 2 hr in the dark at room temperature, acetic acid (final concentration 0.2 M) was added and the solution evaporated. The residue was taken up in a dilute aqueous solution of ^{3}H-rT′ (4×10^{-7} M, 0.46 nCi/μl), which serves as an internal standard [4], so that the volume of the final labelled solution was the same as before addition of borotritide. The solution was then immediately subjected to silica gel TLC and quantitative analysis [3,4].

3. Results and discussion

3.1. *Degradation of a mixture of UpApApGp and m^5CpApApGp.*

A 1:1 mixture of these tetranucleotides was obtained by T_1-RNAase digestion of yeast $tRNA^{Leu}_3$ [6]. Fig. 1A depicts the release of the various nonphosphorylated dialdehyde derivatives from the oligonucleotide chain, as assayed by tritium postlabelling. In accordance with previous results obtained by periodate-phosphatase degradation of ApUpG under

comparable conditions (see fig. 3B of ref. [4], the base composition is reflected by the peak heights of the ^{3}H-labelled alcohol derivatives. Thus, for the examples chosen, the number of A residues to the left of the 3′-terminus directly correlates with the peak height of (^{3}H)-A″. A ratio of 3:2:1 was observed for the peaks obtained from oligonucleotides having three, two, and one A, respectively, adjacent to the 3′-terminal G (compare figs. 1A,B, and C). The sum of the peak heights of m^5C′ and U′, which compounds are derived from the 5′-terminus, approximately equals the peak height of G″, as expected for a 1:1 mixture of the tetranucleotides. Borotritide analysis of nonphosphorylated dialdehyde derivatives released from these compounds thus provides evidence for the sequence U(m^5C)AAG.

Inspection of fig. 1 indicates that there is a defined induction period for the release of the dialdehydes, depending on the distance of the parent nucleoside from the 3′-terminus; these induction periods being approximately 50, 100, 150, and 200 min for the 2nd, 3rd, 4th, and 5th position, respectively, under the conditions chosen.

3.2. *Degradation of CpCpCpApGp (fig. 1B)*

Here it is clear from the peak height of A″ that there is one A adjacent to the 3′-terminal G. The next dialdehyde released is the derivative of C; its peak indicates the presence of two adjacent C residues. The slope of the curve for C″ also suggests that the two C-derivatives are released after their appropriate induction periods and there is no other base interspersed between them. The 5′-terminus is released as cytidine dialdehyde. Since base composition analysis of this compound shows the presence of 3 C, 1 A, and 1 G the sequence of the pentanucleotide may be deduced on the basis of the curves presented in fig. 1B.

3.3. *Degradation of CpCpApApApGp (fig. 1C)*

The height of the A″ peak shows that there are three A residues next to each other adjacent to the 3′-terminus. The fifth position is released as cytidine methylene dialdehyde after an induction period appropriate for its distance from the 3′-terminus; the peak of its reduction product indicates one C residue. The 5′-terminus is released as cytidine dialdehyde. Since base composition analysis of this compound shows the presence of 2 C, 3 A, and 1 G the sequence of the hexanucleotide may be deduced on the basis of the curves of fig. 1C.

3.4. *Comments*

It thus appears that the sequence of oligonucleotides up to a chain length of 6 may be deduced without great difficulty by following the time course of the release of nonphosphorylated dialdehyde derivatives. We have also applied this method to octa- and decanucleotides, e.g. CpApApCpCpApCpC and ApApUpCpUpCpUpUpApGp. Evidence was obtained for complete degradation upon incubation with $NaIO_4$ and phosphatase, e.g. the A residue adjacent to the 5′-terminus of the decanucleotide was clearly recognizable as a late peak of A″. (For reasons discussed below, the nucleoside trialcohol derived from the 5′-terminus itself may not be a reliable indicator for the completeness of the reaction in these examples.) However, since the degradation is extremely sensitive to variations of the $NaIO_4$/nucleotide and enzyme/nucleotide ratios, as shown for ApUpG [4], and the $NaIO_4$/nucleotide ratio in particular decreases during the reaction, it was found difficult to deduce unambiguously the sequence of these large oligonucleotides solely on the basis of an analysis of the nucleoside methylene dialdehydes and nucleoside dialdehydes.

It appeared desirable therefore to explore the possibility of analyzing the degradation process at the level of the oligonucleotide-3′ dialdehydes, see the following paper [5].

As a minor side reaction, nucleoside methylene dialdehydes were found to undergo addition of water to the 4′,5′ double bond when incubated under conditions for periodate-phosphatase degradation [7].

Since the products of this side reaction are nucleoside dialdehydes the identification of the 5′-terminus, which, in contradistinction to non-5′-terminal positions [3], is obtained as a nucleoside dialdehyde, may become difficult in certain cases. As an example, the amount of adenosine dialdehyde, as assayed by borotritide reduction, during degradation of CpCpApApApGp (fig. 1C) was found to increase during the reaction, being about half that of the cytidine derivative (fig. 1C) at later stages of the reaction (6–14 hr). Although this amount is too low to

account for its being derived from the 5′-terminus, we recommend to identify the 5′-terminus by an independent procedure, such as complete digestion of the oligonucleotide with monoesterase-free venom phosphodiesterase. The released 5′-terminal nucleoside is then determined by conversion to its ^{3}H-labelled trialcohol derivative, which may be resolved and identified by published TLC procedures [8].

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