CONTINUOUS DIRECTIONAL DEGRADATION – A NOVEL METHOD FOR SEQUENCE ANALYSIS OF POLYRIBONUCLEOTIDES*

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1. Introduction

Present methods for sequence analysis of polyribonucleotides [1, 2] can be divided into two major groups: i) those which are based on a combination of specific endonuclease cleavage and controlled exonuclease digestion and ii) those which have as their central feature the chemical removal of 3′-terminal nucleotides by aminē-catalyzed β-elimination leading to the formation of an identifiable purine or pyrimidine base [3].

The first approach has found wide application in RNA sequence studies, particularly when coupled with *in vivo* synthesis of ^{32}P-labelled RNA of high specific activity [4]. An essential element of both methods is the necessity of isolating a relatively large number of polynucleotides of shorter chain length than that of the polynucleotide whose sequence is being analyzed.

In order to characterize RNA species that cannot readily be labelled biologically to high specific activity, in particular RNA of human origin, sensitive methods are being developed in our laboratory that do not require *in vivo* labelling [5–7]. We have previously reported on a method for RNA base composition analysis in which tritium label is incorporated chemically following digestion of the polynucleotide to nucleosides [5, 6]. In this communication I now report a novel method for sequential analysis of polyribonucleotides, which also makes use of the chemical introduction of tritium label into RNA derivatives.

The procedure entails the incubation of a polynucleotide in the presence of $NaIO_4$ and alkaline phosphomonoesterase at slightly alkaline pH. Under these conditions a continuous degradation of the polynucleotide takes place which proceeds in the direction from the 3′-terminus to the 5′-terminus and leads to a sequential release of dialdehyde derivatives. The course of the reaction may be conveniently followed by reducing the aldehydes with [^{3}H]KBH_4 to the corresponding ^{3}H-labelled alcohols. This chemical labelling procedure enables one to achieve high sensitivity without biological isotope incorporation. The analysis of the time course of this reaction provides a sensitive means by which the sequence of the polynucleotide can be deduced without requiring the isolation of polynucleotides of shorter chain length.

This method has been studied in model experiments with oligonucleotides as well as tRNA. I shall illustrate the method by choosing adenylyl(3′-5′)uridylyl(3′-5′) guanosine (ApUpG) as a model compound.

2. Experimental

In experiment A (see below, fig.1A) the reaction mixture contained ApUpG (0.001 M), sodium borate, pH 8.0 (0.03 M, the added buffer being 0.1 M $Na_2B_4O_7$/HCl, pH 8.0 at 23°), *E.coli* alkaline phosphomonoesterase (EC 3.1.3.1, 0.2 μg/μl), and $NaIO_4$ (0.006 M). Incubation was at 38° in the dark. In experiment B, the pH of the added buffer was 8.4, and

* This is part XII of a series entitled "Analysis of Nucleic Acid Derivatives at the Subnanomole Level". For part XI, see [7]. Unusual abbreviations: N′ (A′, C′, U′, G′), a nucleoside trialcohol; N″ (A″, C″, U″, G″), a nucleoside methylene dialcohol (see formula II).

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incubation was at 45°. Aliquots were withdrawn during the reaction and stored in a deep-freeze (-72°) until borotritiide treatment.

The reduction was initiated by adding a 5-fold molar excess of [^{3}H] KBH_4 over $NaIO_4$ to each aliquot. (The borotritiide solution used contained 0.1 μmole (20 μCi)/μl.) Incubation was for 2 hr at 23° in the dark. A 20-fold molar excess of 1 N acetic acid over borohydride was then added and the solution evaporated as previously described for the synthesis of ^{3}H-labelled nucleoside trialcohols [5]. The residue was taken up in a small volume of water so that the final solution contained 0.05–0.1 μCi/μl.

The labelled solutions were analyzed by two-dimensional thin-layer chromatography on silica gel in solvents A (first dimension) and B (second dimension), see table 1. Aliquots of the labelled solutions (0.05–0.2 μCi) were applied to the chromatograms and co-chromatographed with marker compounds (see below). Spots were located under UV-light (254 nm) and by fluorography [8]. Radioactive compounds co-chromatographing with A′, U″ and G″ were cut from the chromatograms, extracted with 0.5 M LiCl and assayed as previously described [5].

Nucleoside trialcohols were prepared as described [5]. Nucleoside methylene dialcohols [9] were prepared as follows: Nucleotide dialdehydes were obtained by treating ribonucleoside-5′ monophosphates (0.01 M) with $NaIO_4$ (0.02 M) at 23° for 30 min. Ethylene glycol was added (final conc. 0.1 M) and after 60 min at 23°, the solution was dried in a stream of air. For β-elimination of phosphate, the dry residues were dissolved in 0.1 M $Na_2B_4O_7$/HCl, pH 8.0 (100 μl per μmole of original nucleotide), and the solution was kept at 38° for 6 hr. The resulting methylene dialdehydes (I) were reduced to dialcohols (II) with a 10-fold molar excess of KBH_4 (0.1 M) for 1 hr at 23°:

The solutions thus obtained were used directly as markers for co-chromatography with the labelled samples.

3. Results and discussion

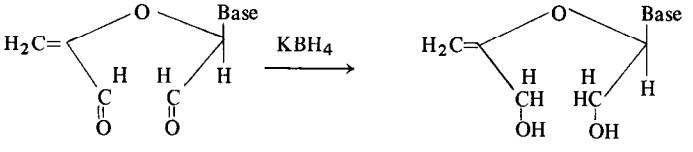
Table 1 presents R_f values of nucleoside trialcohols and methylene dialcohols on silica gel thin layers. All compounds listed in table 1 can be completely separated from each other by two-way chromatography in solvents A and B.

Table 1
R_f values of nucleoside trialcohols and methylene dialcohols on silica gel thin layers*.

Compound	Solvent A	Solvent B
A′	0.54	0.27
C′	0.37	0.19
U′	0.31	0.47
G′	0.19	0.17
A″	0.72	0.52
C″	0.62	0.40
U″	0.48	0.70
G″	0.34	0.33

* Eastman Chromagram sheets no. 6060. Inside dimensions of tank, 27 cm long, by 7 cm wide, by 24 cm deep. Tank sealed by glass contact, not lined for saturation. Ascending chromatography started immediately after pouring solvent into the tank. Origin 2.5 cm from edge. One chromatogram per tank. Temperature 23±1°. Solvent A, acetonitrile/15 N ammonia (3.4:1, v/v). Solvent B, acetonitrile/water/90% (w/w) formic acid (11:1:0.3, v/v). Migration distance of front, 10 cm from origin. The values represent the means of 4 experiments.

Results obtained by chromatographic analysis of the course of the reaction are illustrated in part in fig.1 and can be summarized as follows:



I II

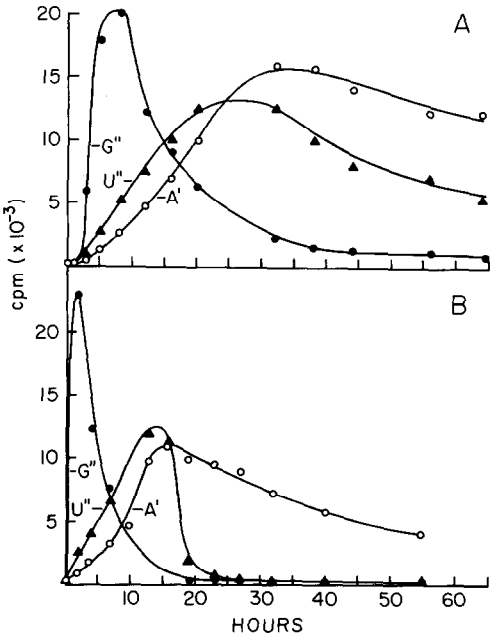


Fig.1. Time course of continuous directional (3'→5') degradation of ApUpG. For reaction conditions and analytical methods, consult text. A = experiment A; B = experiment B.

i) Following borotritiide reduction of the reaction products obtained from ApUpG (see Experimental), three radioactive compounds were detected on chromatograms by fluorographic visualization; these compounds co-chromatographed with G", U" and A'. Similarly, GpA was found to be degraded to a mixture of A" and G'; ApCpC gave C" and A', and A", C" and C' were obtained by incubating CpCpA under the conditions specified for ApUpG. The compound derived from the 5'-terminus was always obtained last.

ii) During the reaction the radioactivity of the methylene dialcohol derived from the 3'-terminal position (G") increased first, reaching its peak after about 8 hr (fig.1A) and 2 hr (fig.1B), respectively. The radioactivity of the dialcohol derived from the second position (U"), rose more slowly, peaking after about 25 hr (fig.1A) and 14 hr (fig.1B), respectively. The 5'-terminal nucleoside was released last and identified following its conversion to the corresponding ^{3}H-labelled trialcohol (A'). The maximum concentration of this compound was found after about 35 hr (fig.1A) and 16 hr (fig.1B), respectively.

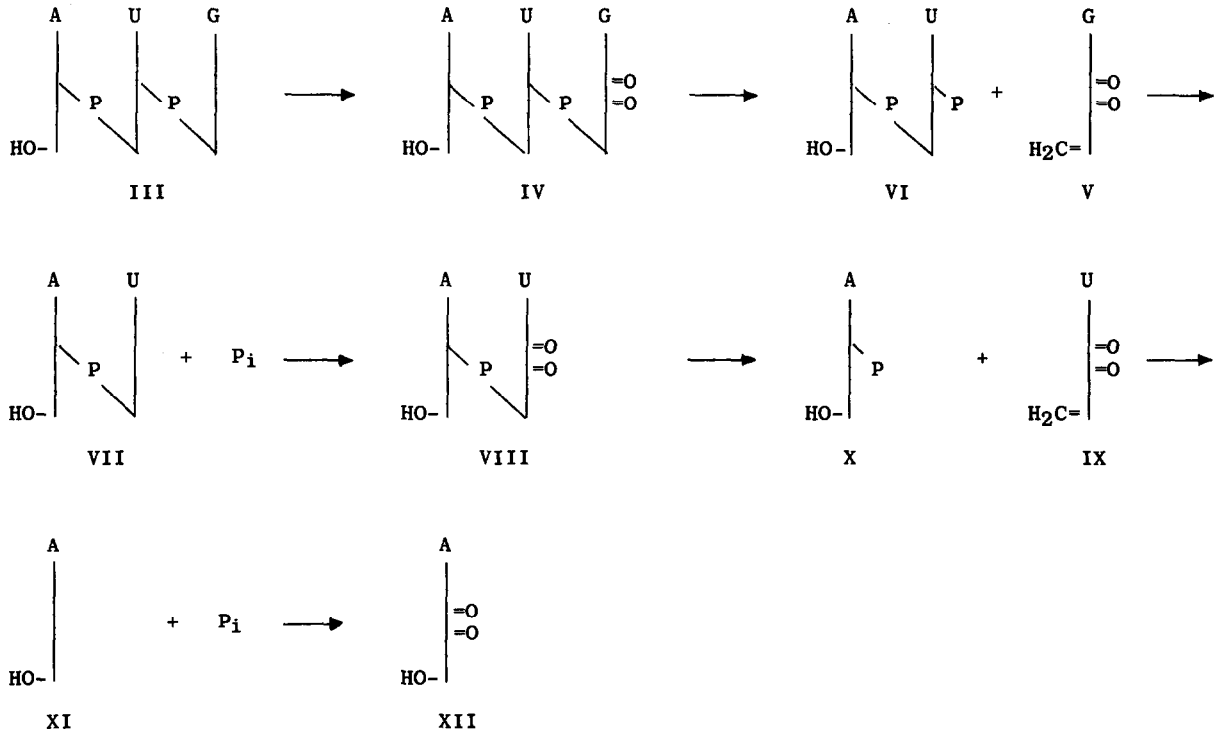


Fig.2. Proposed reaction mechanism for continuous directional degradation of polyribonucleotides, illustrated for ApUpG.

iii) The radioactivity derived from the released products decreased after having reached a maximum suggesting that the parent aldehydes underwent decomposition. The rate of this reaction, which varies for individual compounds (fig.1), appears to be temperature and pH dependent resulting in narrower peaks at 45° and pH 8.4 than at 38° and pH 8.0.

These observations suggest the following reaction mechanism (fig.2): The 3′-terminus of ApUpG (III) is first oxidized by $NaIO_4$ to a dialdehyde derivative (IV), which is β-eliminated as guanosine methylene dialdehyde (V). (This reaction does not require the presence in the reaction mixture of a primary amine [9, 10].) The resulting dinucleoside diphosphate (ApUp, VI) is dephosphorylated by the action of the phosphatase, which retains activity in the presence of $NaIO_4$. ApU (VII) formed in this step is subsequently oxidized to the dialdehyde VIII and the 3′-terminal uridine is β-eliminated as uridine methylene dialdehyde (IX). Following dephosphorylation of Ap (X) to adenosine (XI), the nucleoside is oxidized to a dialdehyde (XII). Treatment with borotritiide converts the various dialdehydes to the corresponding labelled alcohols (G″, U″, A′ and nucleotide dialcohols derived from the nucleotide dialdehydes IV and VIII, see below).

Since, in contrast to other positions of the polynucleotide chain, the formation of the 5′-terminal nucleoside according to this mechanism does not involve a β-elimination step, the 5′-terminus should be converted to the nucleoside relatively soon after the release of its nearest neighbor. This prediction is confirmed by the experimental findings (fig.1).

Further evidence for the postulated reaction mechanism was obtained by chromatographic analysis of alkaline hydrolysates of the ^{3}H-labelled reaction mixtures (10% piperidine, 95°, 2 hr): ^{3}H-labelled guanosine trialcohol (G′) was found to be present in hydrolysates of samples from the earlier part of the reaction; at later stages uridine trialcohol (U′) was also detected in such hydrolysates. These compounds were absent from the original labelled solutions prior to piperidine hydrolysis. These results provide evidence for the presence in the reaction mixtures of oligonucleotides terminating with guanosine dialdehyde (IV) and uridine dialdehyde (VIII), respectively, which upon borohydride treatment are converted to the corresponding dialcohols.

The instability of the aldehydes, which is probably due to aldol condensation, appears to be advantageous since it leads to a narrowing and better resolution of the peaks derived from individual positions of the polynucleotide chain, particularly at the higher temperature and pH (fig.1B). The products of these side reactions have not interfered thus far with the analysis of the radioactive derivatives.

It is evident from fig.1 that the analysis of a few early time points probably will suffice in many cases to deduce the polynucleotide sequence. In order to determine the optimum conditions for continuous directional degradation, particularly as applied to longer oligo- and polynucleotides, a systematic investigation of the kinetic parameters of the various reactions involved is under way in this laboratory. The question of monitoring the reaction in an automatic fashion is also being explored.

Acknowledgements

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