Tritium sequence analysis of oligoribonucleotides: a combination of post-labeling and thin-layer chromatographic techniques for the analysis of partial snake venom phosphodiesterase digests

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ABSTRACT

A tritium derivative method for sequence analysis of polyribonucleotides is detailed, which is based on borotritide reduction of oligonucleotide-3' dialdehydes generated by controlled snake venom phosphodiesterase/alkaline phosphomonoesterase digestion and periodate treatment of time point aliquots of the incubation mixture. Radioactive oligonucleotide derivatives are resolved according to chain length by PEI-cellulose¹ anion-exchange TLC and their 3'-termini identified by techniques described in the preceding paper of this series². The present tritium derivative method is compared with the one described previously².

INTRODUCTION

A tritium derivative method for sequence analysis of polyribonucleotides was described recently², which involves the following steps:

1. Incubation of an oligonucleotide with NaIO_4 and alkaline phosphomonoesterase at pH 8.
2. Treatment of aliquots of the incubation mixture with (^3H)-labeled borohydride.
3. Resolution according to chain length of the labeled oligonucleotide derivatives formed in step 2 by anion-exchange TLC on PEI-cellulose.
4. Digestion in situ of labeled oligonucleotide spots with RNase T_2 to release labeled 3'-terminal nucleoside trialcohols.
5. Resolution of labeled trialcohols by 2-dimensional

partition TLC in the presence of nonradioactive trialcohols as markers for location of compounds.

6. Identification of trialcohols by scintillation counting.

Because incubation of a polyribonucleotide with NaIO_4 and alkaline phosphatase results in a 3'→5' directional degradation process, in which oligonucleotide-3' dialdehydes of decreasing chain length are formed as intermediates⁴, the sequence of the oligonucleotide subjected to this procedure may be deduced by identifying the (³H)-labeled terminal alcohols after reduction of the dialdehydes with tritiated borohydride^{2,5}.

A formal analogy exists between periodate/phosphatase degradation and snake venom phosphodiesterase digestion of oligonucleotides, which is known also to proceed from the 3'-terminus, but with the successive formation of 3'-dephosphorylated oligonucleotides of decreasing chain length as the intermediates⁶. Since these compounds are readily oxidized by periodate to oligonucleotide-3' dialdehydes⁷, i.e. the intermediates of periodate/phosphomonoesterase degradation⁴, it was of interest to examine the feasibility of analyzing the products of snake venom phosphodiesterase digestion by the techniques described² for sequence analysis following periodate/phosphatase degradation. This communication describes experiments indicating that tritium postlabeling of oligonucleotide intermediates in snake venom phosphodiesterase digests can be combined with TLC and *in situ* RNase T₂ digestion, thus providing a highly sensitive method for sequence analysis of nonradioactive oligonucleotides. The 2 isotope derivative methods for sequence analysis will be compared. An octanucleotide, CpApApCpCpApCpC-OH, and a decanucleotide, ApApUpCpUpCpUpUpApGp, which had been isolated from a RNase T₁ digest of tRNA^{Leu 3}_{yeast}^{8,9}, were chosen as model compounds.

EXPERIMENTAL

5'-Nucleotidase activity in commercial snake venom phosphodiesterase (Worthington, code VPH, lot 33P674) was inactivated by acid treatment¹⁰.

Nonradioactive nucleoside dialcohols were prepared as described¹¹. For the preparation of dialcohol derivatives (pN') of nucleoside-5' phosphates, nucleotide (20 mM) was incubated with NaIO_4 (30 mM) in unbuffered aqueous solution for 60 min at 23° in darkness. An aqueous NaBH_4 solution (0.9 M) was then added to obtain an initial borohydride concentration of 0.15 M. After 60 min at 23° in darkness, the solution was cooled to 0° and 1 N acetic acid was added slowly to a final concentration of 0.25 N. The solution was then evaporated and the residue taken up in water (final concentration of pN', 10 mM). These solutions (0.5-1 μl) were used as UV-absorbing markers for co-chromatography with labeled samples.

For sequence analysis, the standard reaction mixture contained: oligonucleotide (about 0.005 mM), magnesium acetate (10 mM), N,N-bis(2-hydroxyethyl)glycine (bicine), pH 8.0 (20 mM), *E. coli* alkaline phosphomonoesterase (Worthington, code BAPF, exhaustively dialyzed against deionized water²; 0.1 $\mu\text{g}/\mu\text{l}$), and acid-treated venom phosphodiesterase (0.01 $\mu\text{g}/\mu\text{l}$). Incubation was at 23° . Five μl of NaIO_4 solution (0.75 mM) was squirted into 10- μl aliquots taken at suitable intervals from the reaction mixture. Oxidation was allowed to proceed for 45-60 min at 23° . The oxidized solutions were then kept at -80° until borotritide treatment. Reduction was initiated by adding a solution of (^3H)- NaBH_4 (New England Nuclear, code NET 023H) or (^3H)- KBH_4 (Amersham-Searle, code TRK 293) to each aliquot, see legend of Fig. 1. (Solutions of these compounds were prepared, assayed and stored as described¹⁷.) After 1 hour at 23° in darkness, acetic acid (final concentration, 0.3-0.5 M) was added (caution: tritium gas is liberated) and the solutions were evaporated in a stream of air under a hood. The residues were taken up in 10 or 20 μl of water. Aliquots of the labeled solutions corresponding to about 0.02 nmole of original oligonucleotide were resolved by anion-exchange TLC on PEI-cellulose in the presence of urea exactly as described previously².

Terminal analysis of the (^3H)-oligonucleotide-3' dialcohols separated according to chain length² was carried out by in situ treatment of spots with RNase T_2 followed by contact transfer

and silica gel TLC².

5'-Termini of oligonucleotides were identified by snake venom phosphodiesterase digestion and tritium labeling as described previously².

RESULTS

The left half of Fig. 1 (A) depicts a fluorogram resulting from applying the present sequencing technique to the octanucleotide CpApApCpCpApCpC-OH. The film shown on the right side of Fig. 1 (B) was obtained in a parallel experiment in which phosphomonoesterase was omitted from the reaction mixture, everything else being the same. The spots from experiment 1A were digested in situ with RNase T₂ and analyzed by 2-dimensional silica gel TLC after contact transfer. Results of this analysis are shown in Table 1. Since the resolution of homologous series of oligonucleotides and their dialcohol derivatives on columns or thin layers of cellulose anion-exchangers in the presence of urea is essentially according to chain length^{2,12,13} and only the 3'-termini of the nucleotide intermediates incorporate tritium upon treatment with periodate and borotritide⁷ the sequence of the octanucleotide, with the exception of the 5'-terminus, can be deduced from the data of Table 1. The 5'-terminus of this nucleotide was shown to be cytidine by the procedure described previously², thus establishing the complete sequence.

Addition of NaIO₄ to the time point aliquots was found to stop almost immediately further degradation by snake venom phosphodiesterase. This appears to be due to enzyme inhibition by NaIO₄ and resistance of terminally oxidized nucleotides to attack by the phosphodiesterase (unpublished observations).

In the absence of phosphomonoesterase (Fig. 1B), nucleoside-5' monophosphates accumulate in the incubation mixture. These compounds, which are converted to (³H)-labeled dialcohol derivatives (pN'-T) upon treatment of the samples with periodate and borotritide, chromatograph on PEI-cellulose in the presence of urea just behind the tetranucleoside triphosphate

Fig. 1

PEI-cellulose TLC of (^3H)-oligonucleotide-3' dialcohols obtained by controlled snake venom phosphodiesterase/phosphatase (A) and snake venom phosphodiesterase (B) digestion of CpApCpCpApCpC-OH, oxidation with NaIO_4 (0.25 mM), and reduction with (^3H)- NaBH_4 (9.0 Ci/mole, about 2.5 mM). Film detection by fluorography.

Table 1

Radioactivity (cpm) of 3'-termini released by in situ RNase T₂ treatment on PEI-cellulose of (3H)-oligonucleotide-3', dialcohol spots following snake venom phosphodiesterase/phosphatase degradation of CpApCpCpApCpC.

(3H)-Tri-alcohol*	Reaction time (min)										Sequence deduced
	0.25	1	2	5	10	15	20	30			
C'8 A'8	5725 30	3974 41	1867 39	92 36							...pC
C'7 A'7	2294 31	4952 44	3471 43	425 28	38 31						...pCpC
C'6 A'6	71 329	113 2634	82 2884	31 944	36 38						...pApCpC
C'5 A'5	172 32	960 92	2773 113	3192 54	39 25						...pCpApCpC
C'4 A'4	107 28	277 41	968 75	5742 53							...pCpCpApCpC
C'3 A'3		79 190	220 3545	50 321	24 25						...pApCpCpApCpC
C'2 A'2			62 61	88 576	86 4812	85 1692	71 783	36 124			...pApApCpCpApCpC

* The subindex indicates the chain length of the oligonucleotide.

dialcohol fraction as shown in Fig. 1B. In the presence of phosphomonoesterase, these derivatives of nucleoside-5' monophosphates cannot be detected in the reaction mixture (Fig. 1A).

Inspection of Fig. 1 also reveals that the rate of degradation is considerably faster in the presence of alkaline phosphatase than in the absence of this enzyme although the octanucleotide did not carry a 3'-terminal phosphomonoester group. Whether this difference in reaction rate is due solely to product inhibition by nucleoside-5' monophosphates or some other effect is currently being investigated.

Labeled compounds traveling between octanucleotide and origin, as well as between di- and trinucleotide and in the heptanucleotide region of the chromatogram, are acid-stable contaminants of the commercial (^3H)- NaBH_4 preparation used. As pointed out previously², commercial (^3H)- KBH_4 usually contains smaller amounts of such acid-stable contaminants than does (^3H)- NaBH_4 .

Table 2 presents data obtained by applying this analytical procedure to the decanucleotide ApApUpCpUpCpUpUpApGp. Count rates shown in this table were measured directly by subjecting PEI-cellulose spots to scintillation counting without elution as described². The identity of the labeled 3'-termini was established on a separate chromatogram by RNase T_2 analysis of spots from the 1-min, 10-min, and 15-min time points. The 5'-terminus of this nucleotide was shown to be adenosine by the procedure described previously².

Sequences determined by the present method were found to agree with those expected on the basis of the sequence of tRNA^{Leu} 3^{8,9}. Periodate/phosphomonoesterase digestion of these oligonucleotides followed by tritium incorporation² (hereafter called Procedure I) was found to give the same sequence data as the present procedure (hereafter called Procedure II). In order to confirm the general applicability of Procedure II, the oligonucleotides DpCpDpApApGp, UpCpypApGp, and ApypUpm⁵CpApAp¹Gp were also analyzed; in each case the results were in agreement with published sequences^{8,9,14} (data

Table 2

Radioactivity (cpm) of (^3H)-oligonucleotide-3' dialcohol spots following snake venom phosphodiesterase/phosphatase degradation of ApApUpCpUpCpUpUpApGp.

Labeled compound*	Reaction time (min)							
	0.25	1	2	5	10	15	20	30
ApApUpCpUpCpUpUpApG'	2538	1458	826	306				
ApApUpCpUpCpUpUpA'	848	1186	1226	932	227			
ApApUpCpUpCpUpU'	316	903	1335	1952	614			
ApApUpCpUpCpU'		256	332	1035	849			
ApApUpCpUpC'		81	273	906	1614			
ApApUpCpU'				399	999			
ApApUpC'				292	1523			
ApApU'					609	1041		
ApA'						2942	4145	1032

* The sequence was determined in a separate experiment, see text.

not shown), as well as with sequence data obtained by subjecting these compounds to Procedure I, see the preceding paper of this series².

DISCUSSION

Current sequencing methods for nucleic acids can be divided into 2 major groups depending on whether or not the molecules to be analyzed are subjected to prior labeling with radioactive isotopes for purposes of structural analysis. Such labeling of nucleic acids, particularly with ^{32}P , may be achieved biologically, i.e. by in vivo incorporation of isotope¹⁵, or enzymatically, i.e. by in vitro incorporation of isotope into complementary RNA or DNA sequences (see a recent review by Salser¹⁶). The in vivo labeling technique as originally proposed by Sanger, Brownlee and Barrell¹⁵ has been instrumental in elucidating many details of RNA structure, particularly in bacterial and viral systems. This approach, however, is frequently not applicable to the structural analysis

of nucleic acids in mammalian tissues¹⁷. In addition, the experimental conditions required for achieving the high specific activity necessary for sequence analysis may give rise to various artifacts^{17,18} which may be difficult to recognize or evaluate. The in vitro transcription ("hybrid") approach to sequence analysis of nucleic acids is not feasible, or may lead to erroneous results, if the template contains modified nucleosides and, in addition, depends on both fidelity and completeness of transcription, factors that have to be evaluated carefully for each incorporation system. Such precautions are not necessary when nonradioactive nucleic acids are subjected directly to sequence analysis. In view of the small amounts of pure nucleic acids available from many, particularly mammalian sources, which preclude utilization of the classical spectrophotometric techniques¹⁹, it is necessary to develop enzymatic and chemical isotope derivative ("post-labeling") methods^{13,17,20-24} for nonradioactive nucleic acids. In contrast to the biological or enzymatic "prelabeling" methods, which entail a remote but distinct possibility of introducing sequence artifacts by the very conditions required for isotope incorporation, there is no such hazard inherent in the postlabeling methods.

The value of labeling techniques for structural characterization of terminal sequences of polynucleotides was first demonstrated by Dulbecco and Smith²⁰ and Khorana and coworkers^{21,22}. However, no nucleic acid has been sequenced thus far solely by postlabeling methods. Since both the tritium derivative method described in the preceding paper of this series², i.e. Procedure I, and the present one, i.e. Procedure II, enable one to deduce the sequences of rather large oligoribonucleotides in a highly sensitive manner it may be possible in the near future to determine the entire sequence of an RNA by postlabeling techniques.

In 1968 RajBhandary described the introduction of tritium label into 3'-terminal positions of nucleotides present in a partial snake venom phosphodiesterase digest of an oligoribonucleotide⁷. This author presented evidence indicating that

this reaction may be a valuable tool in sequence studies on oligonucleotides. As at the time of this work no sensitive method was available for detection of (^3H)-labeled compounds on sheet chromatograms the labeled oligonucleotides were separated by RajBhandary on a DEAE-cellulose column. Procedures I and II allow one to work with minute amounts of oligonucleotides because they combine highly sensitive TLC methods with film detection of tritium by fluorography²⁵. Furthermore, in situ enzymatic liberation of the labeled 3'-termini and the contact transfer technique obviate the preparative isolation of the labeled derivatives. The overall technique is thus rapid and involves a minimum of experimental manipulations

The salient features of Procedure I² and Procedure II may be outlined as follows:

1. As both procedures have as a central element periodate oxidation of ribose residues at 3'-terminal sites of oligonucleotides they are both RNA-specific. In Procedure I, NaIO_4 is an indispensable reagent for the degradation process itself while, in Procedure II, the oxidant plays a secondary role inasmuch as it merely serves to oxidize the oligonucleotide intermediates generated by snake venom phosphodiesterase digestion. This implies an absolute specificity of the digestion process of Procedure I for RNA and RNA fragments whereas snake venom phosphodiesterase is known to degrade oligodeoxyribonucleotides even more rapidly than oligoribonucleotides⁶. In Procedure II, specificity for RNA fragments is thus due solely to the resistance of oligodeoxyribonucleotides to periodate oxidation. This difference between the 2 procedures may be of practical importance, e.g. only Procedure I can be expected to attack specifically the RNA strand of DNA-RNA hybrids leaving the DNA strand intact.

2. While a combination of 3 reactions (enzymatic removal of phosphomonoester groups, oxidation, and phosphodiester bond cleavage by β -elimination) generates the oligonucleotide-3' dialdehyde intermediates in Procedure I⁴ a single reaction (enzymatic hydrolysis of phosphodiester bonds) leads to the

formation of the oligonucleotide intermediates in Procedure II. Digestion by Procedure I is slower than by Procedure II and requires elevated temperature (40-50°) as the chemical reactions involved in Procedure I are slow relative to enzymatic hydrolysis by snake venom phosphodiesterase.

3. Although the intermediates and their derivatives are generated by completely different mechanisms the same compounds, viz. oligonucleotide-3' dialdehydes, are obtained in both procedures so that the same techniques apply to their analysis regardless of the different mechanisms. This implies that the sensitivity of the 2 procedures is similar, see Discussion in the preceding paper of this series². The overall sensitivity is also influenced, however, by the recovery of dinucleotide derivatives, see point 7 below.

4. In both procedures, the nucleotide sequence is deduced by separation of terminally labeled oligonucleotide derivatives according to chain length, in situ enzymatic liberation of (³H)-labeled 3'-terminal trialcohols, contact transfer, and identification of the trialcohols by 2-dimensional TLC and scintillation counting. This technique provides a very direct and unambiguous way of identifying each position of the polynucleotide chain.

5. Procedure II is capable of establishing the sequence to the left of a ribose-methylated nucleoside whereas periodate/phosphomonoesterase degradation will come to a halt after 3'-terminal phosphate has been removed from such a site by the action of phosphatase².

6. Oligonucleotides containing G-rich sequences or certain "hypermodified" nucleosides are known to be rather resistant to snake venom phosphodiesterase digestion²⁶⁻²⁸. In view of the different reaction mechanism, Procedure I is expected to be better suited in some instances to deduce the sequence of such compounds. The more vigorous digestion conditions of Procedure I may, however, lead to side reactions of certain "hypermodified" nucleosides if the substituent is attacked by periodate at elevated temperature.

7. The recovery of "dinucleotides" (dinucleoside phos-

phate dialcohols) was found to be somewhat low in Procedure I depending on the chromatographic conditions². Procedure II consistently affords higher recoveries of these compounds, probably because of the slow action of snake venom phosphodiesterase on dinucleotides (provided they do not carry a 5'-phosphomonoester group^{6,29}). Procedure II thus appears to be slightly more sensitive than Procedure I with regard to the identification of the position adjacent to the 5'-terminal nucleoside.

8. Procedure I is highly reproducible: As mentioned previously², no differences in reaction rates were observed when different batches of commercial, electrophoretically purified alkaline phosphomonoesterase from *E. coli* were compared and there was no indication of endonucleolytic cleavages. (It is possible that the presence of NaIO_4 causes the inactivation of traces of endonuclease during the digestion.) On the other hand, variations in activity and properties of commercial snake venom phosphodiesterase preparations are well known and some batches have been found to be contaminated with endonuclease³⁰. Each preparation of this enzyme has to be assayed, therefore, as to its suitability for partial digestion, and the optimal enzyme concentration should be established in a preliminary experiment. No evidence for endonuclease activity was found in the batch used in the present study (see Experimental) but contaminating 5'-nucleotidase activity had to be removed so as to make the preparation suitable for the identification of 5'-terminal nucleosides.

Thus, in spite of a number of similarities, there are distinct differences between the 2 procedures; it may be hoped that both will be useful complementary tools in sequence studies on RNA in cases where the high sensitivity provided by isotope derivatization is essential for structural characterization of a particular RNA.

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