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A RAPID METHOD FOR DETERMINING NUCLEOTIDE SEQUENCES OF RIBO-OLIGONUCLEOTIDES USING *p*-HYDRAZINOBENZENE [^{35}S]SULPHONIC ACID

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1. Introduction

Present methods for sequence analysis of ribo-oligonucleotides [1,2] fall into two major groups: those based on the sequential β-elimination of 3′-terminal nucleotides [3–16] and those which use a combination of specific endonuclease cleavage and controlled exonuclease digestion [17]. Although β-elimination provides a method for sequencing RNA that cannot readily be labelled biologically to high specific activity, the eliminated nucleoside methylene dialdehydes are unstable. The other method has found wide application in sequence studies on uniformly ^{32}P-labelled RNA, but it requires the isolation and characterization of many oligonucleotides of shorter chain length than the initial polynucleotide.

We now wish to describe a rapid procedure for determining nucleotide sequences of ribo-oligonucleotides using *p*-hydrazinobenzene-[^{35}S]sulphonic acid (^{35}S-*p*-HBSA) as a labelling reagent. This new technique uses endonuclease and controlled exonuclease digestion of the oligonucleotide but oligonucleotides shorter in length than that being sequenced need not be isolated. We describe a simple method to detect the sequential removal of nucleoside 5′-phosphates from the 3′-terminus of the oligonucleotide by an exonuclease.

2. Materials and methods

^{35}S-*p*-HBSA was prepared as described earlier [18,19] from ^{35}S-sulphanilic acid (The Radiochemical Centre, Amersham, England). The oligonucleotides were isolated from a fingerprint [18,20] of a ribonuclease T_1 digest of unlabelled Cowpea Chlorotic Mottle Virus RNA. (The pentanucleotide is No. 14; the tetranucleotide is No. 12 and the trinucleotide is No. 11 on the fingerprint.) All the oligonucleotides were in unbuffered aqueous solution. In order to correlate the results, it was found best to perform the following experiments at the same time using equal amounts of the oligonucleotide.

2.1. *Base composition analysis*

This was done using the ^{35}S-*p*-HBSA labelling method [18,20] with 2 μg of each oligonucleotide. The final volume of the labelled solution was 20 μl, of which 5 μl was used for chromatography on PEI cellulose thin layers (Polygram cell 300, Camlab, Cambridge, England) using a stepwise LiCl method of development.

2.2. *Determination of 5′-terminal residue*

Each oligonucleotide (2 μg) was digested with one unit of snake venom phosphodiesterase in 10 μl water (Calbiochem), 10 μl of 0.01 M $MgCl_2$, and 5 μl of 0.1 M Na_2CO_3–$NaHCO_3$ buffer (pH 9.6), for 8 h at 37°C. The hydrolysate was then labelled with ^{35}S-*p*-HBSA as in the procedure for base composition analysis. Excess ^{35}S-*p*-HBSA was removed by adding alkaline phthalaldehydic acid [20]. The final volume of the labelled solution was 20 μl, of which 5 μl was used for chromatography on PEI cellulose thin layer, using stepwise LiCl development. A blank without any oligonucleotide was similarly processed.

2.3. *Digestion of RNAase T_1-oligonucleotides with RNAase-A*

The oligonucleotide (2 μg) was digested with 1 μg

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pancreatic RNAase-A (Boehringer, Mannheim) in 5 μl water, 5 μg *E. coli* alkaline phosphatase in 5 μl water, 10 μl of 0.1 M Na_2O_3–$NaHCO_3$ buffer (pH 9.6), and 5 μl 0.01 M $MgCl_2$ for 8 h at 37°C. The hydrolysate was then labelled with ^{35}S-*p*-HBSA. The final volume of the labelled hydrolysate was also 20 μl, of which 5 μl was used for chromatography on PEI thin-layer cellulose. A blank without oligonucleotide was similarly processed.

2.4. *Sequential 3′ → 5′ cleavage of the oligonucleotides with snake venom phosphodiesterase*

The oligonucleotide (5 μg) in 200 μl unbuffered aqueous solution was incubated for 15 min at 50°C with 20 μl 0.01 M $MgCl_2$, 25 μg *E. coli* alkaline phosphatase in 25 μl water and 10 μl 0.1 M sodium carbonate buffer (pH 9.6). One unit of snake venom phosphodiesterase in 10 μl water was added and incubated at 38°C in a water bath. At 0, 10, 20, 30, and 45 min 50 μl samples were transferred to small test tubes and immediately heated to 90°C in a water bath for 10–15 min to deactivate the phosphodiesterase to prevent further cleavage of the oligonucleotide. An additional 5 μg of the alkaline phosphatase was then added to each of the samples which were incubated at 38°C for 30 min to dephosphorylate the nucleoside 5′-phosphates.

Each sample was next labelled with ^{35}S-*p*-HBSA. Excess ^{35}S-*p*-HBSA was removed by adding alkaline phthalaldehydic acid [19]. The final volume of the labelled solution was 20 μl, of which 10 μl was used for chromatography on PEI cellulose thin layers. A blank without oligonucleotide was processed like the other samples. The results for the tetranucleotide and pentanucleotide are in figs 1 and 2.

3. Results

3.1. *Nucleotide sequence of the trinucleotide*

The base composition was found to be AGU and the 5′-terminal residue of this trinucleotide to be adenosine, so the sequence AUG is suggested and was confirmed by the result of the simultaneous RNAase-A/phosphatase digestion which released guanosine.

3.2. *Nucleotide sequence of the tetranucleotide*

The base composition was C_2GU and the 5′-terminal mainly cytidine but with some uridine. When treated with RNAase-A and alkaline phosphatase guanosine, cytidine and uridine were released in the ratio 1:2:1. Results of the snake venom phosphodiesterase sequential cleavage (fig.1) are explained as follows: initially the radioactivities of the labelled guanosine and cytidine residues are about equal, but since the tetranucleotide came from a RNAase T_1

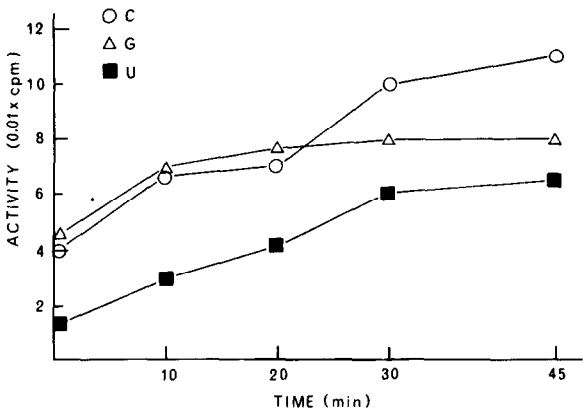

Fig.1. Time course of the 3′ → 5′ sequential release of nucleosides from the tetranucleotide CUCG by a mixture of snake venom phosphodiesterase and *E. coli* alkaline phosphatase (for procedure see text). C, G, and U refer to labelled nucleosides.

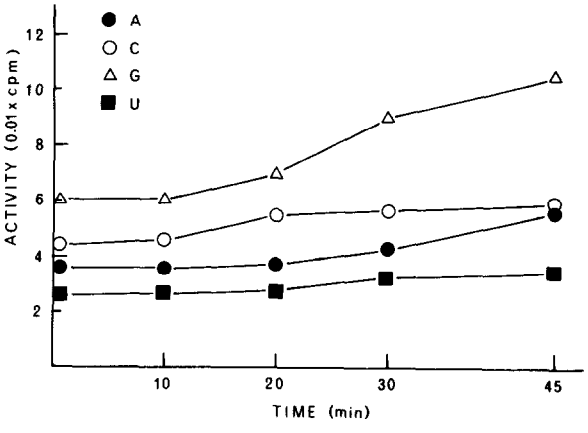

Fig.2. Time course of the 3′ → 5′ sequential release of nucleosides from the pentanucleotide AUACG by a mixture of snake venom phosphodiesterase and *E. coli* alkaline phosphatase (for procedure see text). A, C, G and U refer to labelled nucleosides.

digestion of RNA, guanosine must be the 3′-terminal residue and so the first nucleoside released, and cytidine must be next to guanosine and so the second residue released. The slow release of uridine indicates that it is near the 5′-terminus of the tetranucleotide. This order of release is maintained up to the 20 min sample after which the radioactivity of labelled cytidine rises above that of guanosine. Most of the tetranucleotide molecules at this time have lost their guanosine residue leaving a trinucleotide with a 3′-terminal cytidine residue which is then being removed rapidly. The cleavage of the trinucleotide first to the dinucleotide and then to nucleosides results in the release of two cytidine residues and one uridine residue, hence the steep rise in the radioactivity of labelled cytidine above that of guanosine in the 20–30 min interval. These data indicate that the tetranucleotide has the sequence CUCG. As the 5′-terminal residue estimation gave some uridine, this 'tetranucleotide' must be a mixture of the isomers: CUCG and UCCG, the former being predominant.

3.3. *Nucleotide sequence of the pentanucleotide*

The base composition of this was found to be A_2CGU, the 5′-terminal residue is adenosine and only guanosine was released when the pentanucleotide was digested with a mixture of RNAase-A and alkaline phosphatase. The partial nucleotide sequence of the pentanucleotide is therefore A(ACU)G but a pyrimidine must be on the 5′-side of guanosine. Near zero time of phosphodiesterase cleavage of the pentanucleotide (fig.2), labelled guanosine predominates followed by cytidine, adenosine, and uridine, in that order which is retained throughout the experiment. From this the nucleotide sequence is either AUACG or AAUCG. The latter sequence is ruled out because if such a pentanucleotide were digested with a mixture of RNAase-A and alkaline phosphatase, guanosine and cytidine would have been released, whereas only guanosine was released from the pentanucleotide. The correct sequence of the pentanucleotide is AUACG.

4. Discussion

The 5′-terminal residue of an oligonucleotide is released as a nucleoside by digesting the oligonucleotide with snake venom phosphodiesterase. With ^{32}P-labelled oligonucleotides this residue will not be detected whereas, in the ^{35}S-*p*-HBSA labelling procedure, this residue can be directly labelled and identified. This ^{35}S-*p*-HBSA identification of 5′-terminal residue is also useful because it can indicate the heterogeneity of an oligonucleotide preparation.

Sequential 3′ → 5′ degradation of an oligonucleotide is widely employed in nucleic acid sequence studies [13,21]. But different from previous methods, the ^{35}S-*p*-HBSA procedure uses a mixture of snake venom phosphodiesterase and alkaline phosphatase to cleave an oligonucleotide sequentially, releasing nucleosides which are detected and estimated by labelling with ^{35}S-*p*-HBSA. It is unnecessary to isolate and characterize the partial degradation products of the oligonucleotide.

Unlabelled oligonucleotides up to pentanucleotides have been sequenced easily using this new procedure. The development of another procedure for sequencing longer oligonucleotides using ^{35}S-*p*-HBSA as the labelling reagent is currently in progress.

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