A NEW METHOD FOR PARTIAL DIGESTION USEFUL FOR SEQUENCE ANALYSIS OF POLYNUCLEOTIDES

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1. Introduction

From the structure of the end products, obtained by digestion of a polynucleotide with ribonuclease T_1 and pancreatic ribonuclease it is usually impossible to reconstruct a unique sequence. Several methods have been used to complete the relative ordering of the resulting oligonucleotides. Very often partial digest with the same base-specific ribonuclease allows the isolation of a series of fragments, whose structure can be unambiguously established by further enzymatic hydrolysis [1, 2]. Under normal digestion conditions, however, the afore mentioned enzymes suffer from the disadvantage that they attack preferentially the single-stranded regions in the RNA, so that often it is impossible to obtain all the required overlapping sequences.

At pH 4, on the other hand, the normal secondary structure of MS2 RNA is almost entirely lost (unpublished analytical ultracentrifuge data from H. Slegers). In addition, by carrying out the partial digestion at room temperature or at 37° instead of at 0°, the remaining secondary structure can be further eliminated or loosened. For this purpose, a ribonuclease of low but very specific activity is useful. Goldstein [3] has previously shown that CM-RNase* [4] has lost most of the original nuclease activity, but is still able to depolymerize 5 S RNA (at pH 7.4). In this paper we report the mode of action at pH 4 of this CM-RNase on a polynucleotide derived from MS2 RNA. Conditions are described which permit the desired degree of specific degradation, independent of the secondary structure of the polynucleotide.

2. Methods

^{32}P-Labelled MS2 RNA was partially digested with ribonuclease T_1 and fractionated by electrophoresis on a polyacrylamide gel slab at pH 8. Further purification was obtained by a second electrophoretic separation at acid pH (fig. 1). The polynucleotide used in the present study was $B_9\ z_1$, which has a chain length of 79 monomers (the system for identifying the bands is described in [6].

The polynucleotide was digested with ribonuclease T_1 and pancreatic ribonuclease, and the resulting oligonucleotides were separated and sequenced essentially as described by Sanger and coworkers [7, 8]. These results will be presented elsewhere.

Pancreatic ribonuclease ϵ-carboxymethylated at the lysine in position 41 (CM-RNase), was kindly provided by Mr. F. Molemans and had been prepared as described by Heinrikson [4].

The RNA was incubated with this enzyme in a solution containing 0.05 M Na-acetate, 0.002 M EDTA and 0.04% gelatine. The resulting fragments were separated either by electrophoresis on cellulose acetate at pH 3.5 in the first dimension, followed by homochromatography on DEAE-paper in the second dimension (digests b and d), or by two-dimensional electrophoresis on a gel slab (digest c) [6].

* Unusual abbreviations: CM-RNase is ϵ-carboxymethyl-lysine-41-pancreatic ribonuclease A.

Fig. 1. Electrophoretic separation of band B_9 at pH 3.5 in the presence of 7 M urea. The RNA, extracted from band B_9, was dissolved in 20 μl solution, containing 400 μg carrier yeast RNA, 7 M urea, sucrose and xylene cyanol FF. The mixture was run overnight at 600 V on a 10% polyacrylamide gel containing 0.025 M citrate buffer pH 3.5 and 6 M urea [5]. The arrow (X) shows the position of the dye marker.

3. Results and discussion

The structure of all the small fragments, obtained by partial digestion, could be established by further hydrolysis with ribonuclease T_1 and with pancreatic ribonuclease. The sequences of the CM-RNase fragments, combined with the structure of the T_1-oligonucleotides, provided sufficient information to build up a unique nucleotide sequence for the polynucleotide $B_9\ z_1$, which is presented in fig. 2.

The analysis of the products obtained by digestion under the most severe conditions used (fig. 2b), shows that all pyrimidine nucleotide–Ap links are cleaved. Some pyrimidine-nucleotide–Gp bonds are hydrolyzed as well, but almost no pyrimidine–pyrimidine links are split. These results are consistent with the work of Witzel [9], who showed that the relative velocity constants for pancreatic ribonuclease digestion at neutral pH were CpA, 270; UpA, 110; CpG, 45; CpC, 22; UpC, 3.6; CpU, 2.5; and UpU, 1. Under milder conditions (fig. 2c and d), the preference for CpA and (to a lesser extent) UpA is further emphasized.

Although we believe that at neutral pH part of this polynucleotide is folded in a secondary structure, there is no detectable influence of this on the partial products obtained at 37° and pH 4. Possibly, at 0° there might still be some effect (fig. 2d).

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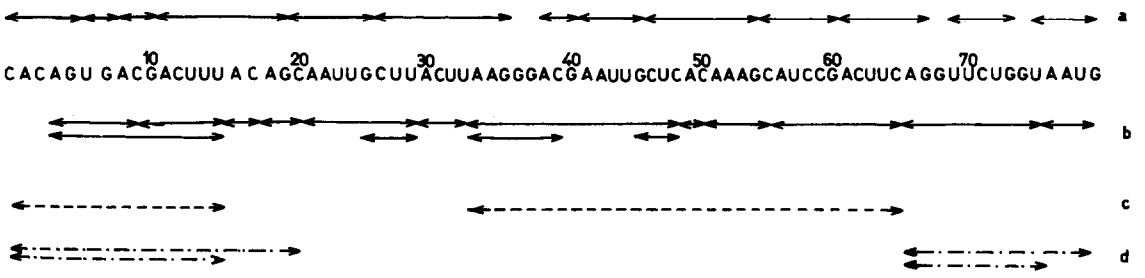

Fig. 2. The sequence of the polynucleotide $B_9\ z_1$. (a) Products, obtained by complete digestion with ribonuclease T_1, were separated by electrophoresis on cellulose acetate at pH 3.5, followed by electrophoresis in 7% formic acid on DEAE-paper. (b) Major products obtained by digestion with CM-RNase at an enzyme to substrate ratio of 1/25 (w/w) during 30 min at 37°. (c) Two fragments obtained in good yield after digestion at an enzyme to substrate ratio of 1/70 (w/w) during 20 min at 37°. (Some other products were also present but were not further characterized.) (d) Fragments obtained by digestion at an enzyme to substrate ratio of 1/70 (w/w) during 30 min at 0°. Much material did not move in the homochromatography system, and presumably consisted of undegraded polynucleotide together with most of the middle region. The partial products from digests b and d were characterized by digestion with RNase T_1 and pancreatic RNase followed by one-dimensional fractionation on DEAE-paper at pH 1.5 and 1.9 respectively. The largest product of digest c was characterized by two-dimensional fingerprinting.

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