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Rapid RNA sequencing: nucleases from Staphylococcus aureus and Neurospora crassa discriminate between uridine and cytidine

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ABSTRACT

Using end-labelled RNA, significant changes in base specificity of three nucleases have been detected under defined conditions. Staphylococcus aureus nuclease at pH 3.5 without Ca^{++} cleaves all Pyr-N bonds more uniformly and efficiently than RNase A, without any preference for Pyr-A bonds. At pH 7.5 in 10 mM Ca^{++} this enzyme cleaves all N-C and N-G bonds slowly, whereas N-U and N-A bonds are hydrolyzed rapidly. Hence, the base at the 3'- or at the 5'-side of a phosphodiester bond can determine the base specificity of S. aureus nuclease. - In absence of urea, Neurospora crassa endonuclease cleaves all phosphodiester bonds, but leaves all C-N bonds intact in 7 M urea. - RNase U_2 at pH 3.5 cleaves A-N bonds more efficiently than at pH 5.0.

INTRODUCTION

Rapid methods involving polyacrylamide gel electrophoresis as developed by Donis-Keller et al. (1), Simoncsits et al. (2) and others (3) have become indispensable for RNA sequencing. Using exclusively in vitro labelling procedures, we have recently established the primary and secondary structure of potato spindle tuber viroid, a pathogen formed by a covalently closed ring of 359 ribonucleotides (4,5). A problem in establishing RNA sequences during this work was occasionally to differentiate uridines and cytidines in oligopyrimidine sequences, in spite of the availability of RNase Phy I (6), which leaves mainly C-N bonds intact (2,7). Consequently, two-dimensional homochromatographic separation and sequence analysis of oligonucleotides from complete RNase T_1 and RNase A digests seemed still to be essential, in addition to the new rapid methods. Other possibilities are the use of slower polyacrylamide gel sequencing procedures (8,9) or to apply a combination of

"primary" and "secondary" postlabelling (10). New alternative enzymatic approaches for uridine and cytidine discrimination using nucleases from Staphylococcus aureus and Neurospora crassa are presented in this report.

MATERIAL AND METHODS

Nuclease from Staphylococcus aureus was from Worthington; although this enzyme appeared electrophoretically homogeneous, the pyrimidine specific nuclease activity observed at pH 3.5 was not found in preparations from another commercial source. Endonuclease from Neurospora crassa, RNase A and calf intestinal alkaline phosphatase were from Boehringer, Mannheim. The ammonium sulfate suspensions of alkaline phosphatase and N. crassa nuclease were centrifuged and the precipitated enzymes dissolved in appropriate volumes of water. RNase T_1 and RNase U_2 were obtained from Sankyo, Tokyo. Nuclease P_1, mung bean nuclease and RNase C were from PL-Biochemicals; silkworm nuclease from Seikagaku Kogyo, Tokyo, was a gift of Prof. S. Nishimura, RNase Phy I from Physarum polycephalum was a gift of Prof. J.P. Bargetzi; RNase from Xenopus laevis oocytes was prepared according to Berridge et al. (11).

50 µg rabbit liver 5S rRNA (10) were treated with 0.1 U calf intestinal phosphatase according to Silberklang et al. (12) in 100 µl 100 mM Tris-HCl, pH 8.0. The phosphatase was inactivated by incubation at 50°C in 7 mM nitrilotriacetic acid for 20 min. The dephosphorylated RNA was recovered by ethanol precipitation, 5'-^{32}P-labelled with T4-kinase as described (4) and purified by two-dimensional polyacrylamide gel electrophoresis (13,14) in order to remove degradation products. 5'-^{32}P-labelled 5S RNA was recovered from excised gel pieces by electrophoretic elution (15) in presence of 50 µg carrier tRNA.

For sequence analysis on polyacrylamide gel, the following enzymes were used for controlled digestions:

a) RNase T_1 and RNase A as described (1).

b) RNase U_2 either as described (1) with 0.5 or 0.05 U RNase per µg RNA in 20 mM sodium citrate, pH 5.0, or in sodium citrate at pH 3.5 with 0.02 U RNase per µg RNA (other conditions as in

ref.1).

c) RNase Phy I as in ref.4, but incubation was for 60 min at 50°C with 0.7 or 0.07 mU RNase per µg RNA.

d) Nuclease from Staphylococcus aureus: 1 to 5 x 10^4 cpm of the 5'-^{32}P-labelled RNA with 4 µg carrier tRNA were digested for 15 min at 50°C in 5 µl 0.03 % xylene cyanol and bromophenol blue, 20 mM sodium citrate, pH 3.5, with 15 or 1.5 U nuclease. An alternative digestion in 10 mM $CaCl_2$ was performed as above except that it was done in 20 mM Tris-HCl, pH 7.5.

e) Nuclease from Neurospora crassa: 1 to 5 x 10^4 cpm 5'-^{32}P-labelled 5S RNA with 4 µg carrier tRNA were incubated for 15 min at 50°C with 5 or 0.5 µg RNase in 5 µl 0.03 % xylene cyanol and bromophenol blue, 20 mM Tris-HCl, pH 7.5 in 7 M urea, or with 0.5 or 0.05 µg nuclease in absence of urea. In this latter case the digest was transferred onto 3 mg urea before loading it onto the sequencing gel.

Controlled acid hydrolysis: labelled RNA as above was heated in 5 µl 0.01 N H_2SO_4 for 1 min at 100°C. This digest was pipetted onto 3 mg urea and 1 µl dye solution before gel electrophoresis.

Fractionation was performed on thin (0.35 mm) 20 % polyacrylamide gels (16) at 1200-1500 V without pre-electrophoresis.

RESULTS

Controlled digests of 5'-^{32}P-labelled 5S RNA produced by RNases T_1 and U_2, which identify guanosines and adenosines, respectively, RNase A, which should identify the pyrimidines, and nuclease from Staphylococcus aureus are presented in Fig.1.

Two properties of the Staphylococcus enzyme become evident:

a) At pH 3.5 without $CaCl_2$, this enzyme cleaves phosphodiester bonds as expected for RNase A, however, polypyrimidine stretches are cleaved more efficiently, and there is no preference for Pyr-A bonds (lane S).

b) At pH 7.5 in 10 mM $CaCl_2$, the base specificity of the S. aureus nuclease appears to be completely different: The base at the 3'-side and not, as at pH 3.5, the base at the 5'-side determines the cleavage rate of a phosphodiester bond. As can

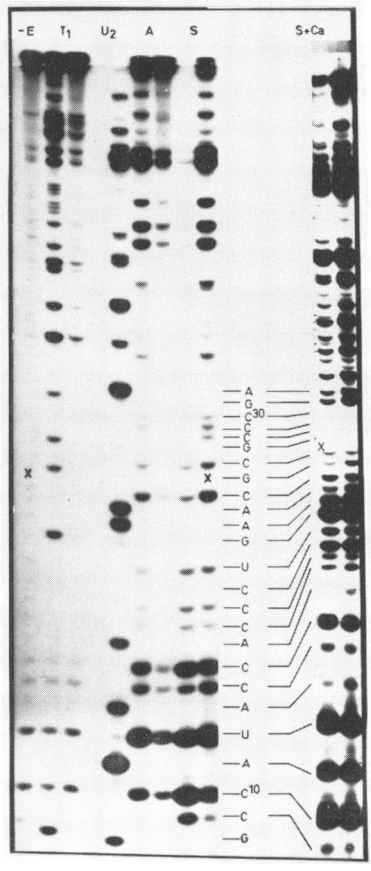

Fig.1. Autoradiogram of controlled digests of 5'-^{32}P-labelled rabbit liver 5S RNA analyzed on 20 % polyacrylamide gel. Lanes: -E, no enzyme; T_1, U_2, A, incubation with corresponding nucleases at two different enzyme/substrate ratios; S (S+Ca), incubation with Staphylococcus nuclease at pH 3.5 (at pH 7.5 in 10 mM Ca^{++}). Lanes -E to S and S+Ca derive from two different polyacrylamide gel electrophoreses; the lines between lanes S and S+Ca connect identical oligonucleotides. It should be noted that C_{19} is not represented as a band in lanes A and S. 5S RNA nucleotides are counted from the 5'-end as in ref.34. 5S rRNA from KB cells (34) and rabbit liver (35) obviously have the same nucleotide sequence. X, position of the xylene cyanol dye marker.

be seen, strong bands are generated by cleavage of the phosphodiester bonds C-A_{11}, A-U_{12}, U-A_{13}, C-A_{16}, C-U_{20}, G-A_{22}, A-A_{23}, G-A_{32}, and A-U_{33}. Consequently, a strong band indicates that the nucleotide following in the sequence (read from bottom to top of the gel) is either U or A (lane S+Ca).

Fig.2 compares controlled digests of 5'-^{32}P-labelled 5S RNA produced by RNase T_1 and RNase U_2, respectively, and RNase Phy I, which leaves mainly C-N bonds intact and helps to differentiate U and C. Acid hydrolysis generates a ladder of all possible products. Neurospora crassa nuclease digestion without urea results in a ladder (lane N, Fig.2) similar to that produced by acid hydrolysis (lane H^+). Interestingly, however, this nuclease does not cleave C-N bonds in presence of 7 M urea (lane N+U).

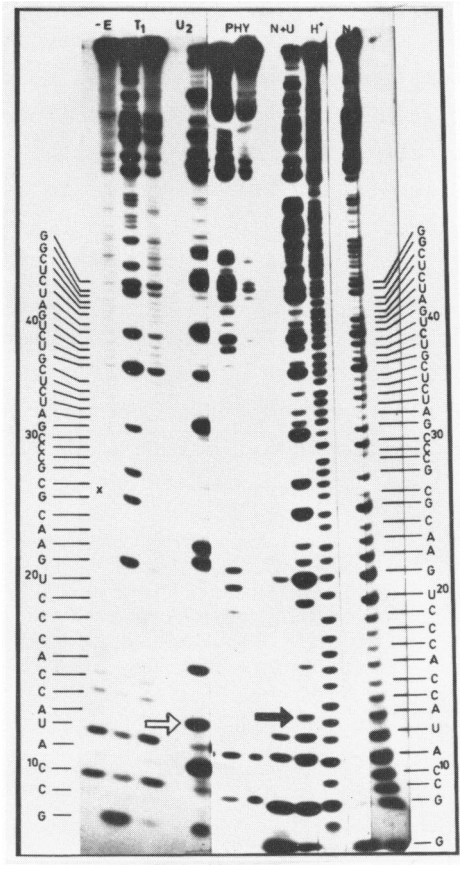

Fig.2. Autoradiogram of controlled digests of 5'-labelled 5S RNA on 20 % polyacrylamide gel. Lanes: -E, T_1, U_2, as in Fig.1.; PHY: digestion with RNase Phy I at two different enzyme levels; N+U, incubation with Neurospora nuclease at pH 7.5 in 7 M urea; H^+, acid hydrolysis of RNA; N, digestion with Neurospora nuclease at pH 7.5 without urea. - Due to the absence of a phosphate at their 3'-ends, oligonucleotides produced by Neurospora nuclease move slower in the gel (nucleoside symbols at the right) than corresponding fragments generated by the other RNases (nucleoside symbols at the left). To give an example, the white arrow points at the band representing A_{13} in lanes -E to PHY and H^+, whereas the black arrow identifies the A_{13} band in Neurospora digests, lanes N+U and N.

For the interpretation of these Neurospora nuclease digests it is most important to bear in mind that this enzyme, in contrast to the other nucleases used in this experiment, creates 5'-^{32}P-

labelled oligonucleotides lacking a 3'-phosphate (17). Consequently it has to be considered when reading sequences that these oligonucleotides move slower in the gel so that the Neurospora ladder is displaced upwards by about one nucleotide unit.

Fig.3 shows that some A-N bonds are resistant to RNase U_2 cleavage at pH 5.0 (lane $U_2 5$), but are quite accessible at pH 3.5 even at a much lower enzyme to RNA ratio (lane $U_2 3.5$).

Several other nucleases failed to be useful for sequence analysis on polyacrylamide gels under a variety of conditions: RNase C from human plasma (2,7,18), RNase from Xenopus laevis (11), nuclease P_1 (19), silkworm nuclease (20) and mung bean nuclease (21). We also tried to protect certain phosphodiester bonds by specific pyrimidine modification with methoxyamine (22,23)

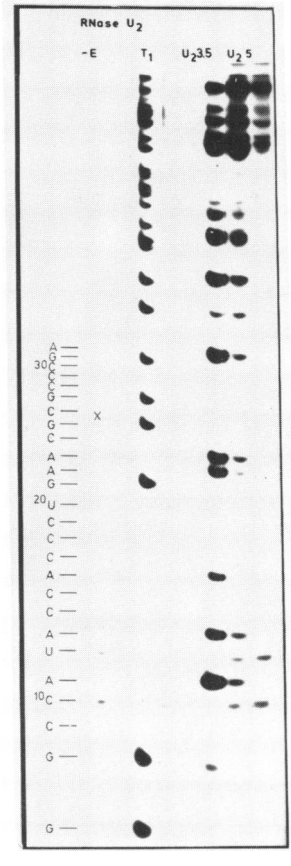

Fig.3. Autoradiogram of partial digests of 5'-labelled 5S RNA. Lanes: -E and T_1, as in previous Figures; $U_2 3.5$ ($U_2 5$), digestion of RNA with RNase U_2 at pH 3.5 (at pH 5.0). Note that the bands representing C_{10} and U_{12} are already present in lane -E.

or Girard-P reagent (24) without success since even in 7 M urea no homogeneous and quantitative base modification was achieved.

DISCUSSION

Controlled digestion of end-labelled RNA with RNase T_1 and RNase U_2, respectively, identifies G and A unequivocally on sequencing gels. Localization of pyrimidines with RNase A is far less reliable due to a strong preference of this enzyme for Pyr-A bonds (7,25). More reliable results are achieved with a pyrimidine-specific RNase from a B. cereus mutant (13).

We have demonstrated here, that commercially available nucleases from Neurospora crassa and Staphylococcus aureus can be used to identify pyrimidines and to discriminate between U and C.

At pH 3.5 without Ca^{++}, Staphylococcus nuclease shows a new and surprising pyrimidine specificity, i.e., the base at the 5'-side of a phosphodiester bond determines the cleavage rate. It should be mentioned that in presence of 10 mM Ca^{++}, however, the above base specificity is lost and all phosphodiester bonds are cleaved (not shown).

A comparison of degradation patterns (Fig.1) shows that Staphylococcus nuclease at pH 3.5 (lane S) identifies pyrimidines more efficiently than RNase A. There is no evidence that this specific activity derives from a contaminating nuclease since three different batches of S. aureus nuclease from Worthington used in these experiments were homogeneous, as evidenced by electrophoresis on cellulose acetate sheets (26), using 0.5 M Trisborate buffer, pH 7.5, and in SDS-polyacrylamide gels (27) (not shown). However, this activity was absent in S. aureus nuclease preparations from another commercial source.

Although special methods have been developed (8-10,28) and in spite of the availability of RNase Phy I, enzymatic discrimination between U and C upon reading sequences from gels is still a problem (2,7,10,25,29-31). In contrast to its pyrimidine specificity displayed at pH 3.5, Staphylococcus nuclease exhibits its known, completely different base specificity if used at pH 7.5 in 10 mM Ca^{++} (32). In this case the base at the

3'-side of a phosphodiester bond determines the hydrolysis rate and thus allows one to distinguish U and C: A strong band on the sequencing gel indicates that the nucleotide following in the sequence, i.e. that at the 3'-side of the cleaved phosphodiester bond, is A or U (lane S+Ca). This unique specificity of S. aureus nuclease helps to identify U and C even in those cases where this is difficult to achieve due to bands (C_{10} and U_{12} in Fig.1) already present in the control (-E) resulting from non-enzymatic Pyr-A cleavage (25).

C and U identification via controlled S. aureus nuclease degradation of end-labelled RNA may sometimes be ambiguous since a classification of weak (for C and G) and strong bands (for U and A) is required. Fig.2 demonstrates that nuclease from Neurospora crassa provides an additional and independent tool for C and U localization in RNA. Without urea in the digest we find cleavage of all phosphodiester bonds (lane N). This is in contrast to results obtained under similar conditions from homopolymer digestions or from end group analysis of oligonucleotides in RNA digests (33) indicating resistance of C-N bonds. As can be seen, under our conditions base specificity of N. crassa nuclease depends upon the presence of 7 M urea only (lane N+U). The U-N bonds are cleaved uniformly not only in oligopyrimidine sequences (Fig.2), but also in oligo U stretches (not shown). - In contrast to a newly developed direct chemical method for sequencing RNA (36), the enzymatic approach described here is applicable for 5'-labelled RNA.

In summary, a major result of this study is the observation that S. aureus and N. crassa nucleases, respectively, display different base specificities under different defined conditions. The application of these findings for rapid sequencing of end-labelled RNA provides a new approach to pyrimidine identification and discrimination. An application of these nucleases for sequencing DNA should be considered.

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