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TWO PLANT NUCLEASES AS TOOLS FOR STRUCTURAL ANALYSIS OF RNA

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Key Word Index—*Secale cereale*; rye; *Triticum vulgare*; wheat; Gramineae; single-strand-specific nucleases, RNA secondary structure studies, CopA RNA

Abstract—Two distinct plant nucleases. nuclease I from nucleoplasm of rye germs—Rn enzyme [1] and nuclease I from wheat chloroplasts—Wch enzyme [2] have been used to investigate the secondary structure of the small RNA molecule–CopA RNA in solution. Both nucleases are good tools for such studies: they can act on RNA within the physiological pH range and in commonly used TMN buffer. Rn nuclease is base non-specific and is capable of cleaving all four phosphonucleotide bonds in non-base-paired regions while double-stranded structures are completely resistant to the nucleolytic attack even with more extensive hydrolysis. The enzyme is very stable and can retain its activity for many years Wch nuclease has a clear preference for pyrimidines in non-base-paired regions.

INTRODUCTION

The secondary structure of RNA is strictly connected with its biological function For this reason it is very important to have a variety of good tools for structural analysis of RNA molecules. Most physical and chemical methods used for RNA structure studies provided general information about this structure and were unable to pinpoint helical regions and their lengths within a sequence [3]. A few years ago single-strand-specific nuclease S1 was used in a new method of secondary structure mapping of two RNA species. yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Glu_2}$ [4]. The method is very simple, but sometimes inconvenient in use because of the low pH optimum of this nuclease, which may cause depurination of nucleic acids in extended digests. In addition to S1 nuclease a few other RNases were used for RNA structural studies. Those enzymes are usually base-specific, so the RNA molecule structure should be analysed with the use of at least several enzymes at a time.

We decided to test two single-strand-specific nucleases from higher plants: nuclease Rn from nucleoplasm of rye germs [1] and nuclease Wch from wheat chloroplasts [2] to analyse the known secondary structure of the small RNA molecule–CopA RNA–which is involved in the control of replication of plasmid R1 [5]. Both those enzymes were shown to have a high degree of specificity toward single-stranded regions in native nucleic acids. Both of them have a higher pH optimum than S1 nuclease and can act at pH ranges close to physiological.

RESULTS

Limited digestion of CopA RNA with Rn nuclease from rye germ nucleoplasm

Synthesized *in vitro* 5'-end labelled CopA RNA was subjected to partial digestion with single-strand-specific Rn nuclease from rye germ nucleoplasm. The products of reaction were analysed on 12 and 20% polyacrylamide sequencing gels containing 7 M urea. In addition CopA RNA was subjected to partial digestion of other structure specific enzymes: single-strand-specific ribonuclease T1 under denaturing and nondenaturing conditions, and double-strand-specific cobra venom nuclease (V1). Limited alkaline hydrolysis of CopA RNA provided a complete set of oligonucleotides by cleavage at every phosphodiester bond. The exact positions at which Rn nuclease cleaves the RNA molecule were mapped by comparing the locations of RNA-generated bands with bands of known lengths produced by alkali and base-specific RNase T1. The digestion patterns obtained are seen in Fig 1. The data were compared with the computer model for the CopA RNA molecule [6] and with the published secondary structure of this molecule [5]. Rn nuclease shows high specificity toward single-stranded regions in the RNA native molecule, but does not show base specificity and can cleave between any four nucleotides. Thus, the enzyme attacks all phosphodiester bonds in the smaller loop of CopA RNA (positions 9–12). Additionally Rn nuclease cleaves CopA RNA at position 8 suggesting, that this residue is also non-base-paired, at least to some extent. The double-stranded part of the CopA molecule (stem I) is resistant to the nucleolytic attack of the Rn enzyme, except position 20 where the nuclease cleaves the phosphodiester bond. That may suggest that this position has a loosened secondary structure (structural breathing). Rn nuclease cleaves phosphodiester bonds in the single-stranded spacer region (positions 21–34). Phosphodiester bonds following guanosines at positions 30, 32, 33 and 34 are very poorly cleaved by Rn nuclease, which may suggest, that this region is partially double-stranded, for example by pairing with uridylate residues from the 3' tail. This is in good agreement with the observation, that double-strand-specific V1 nuclease cleaves phosphodiester bonds of U83 and U84 from the 3' tail of CopA RNA

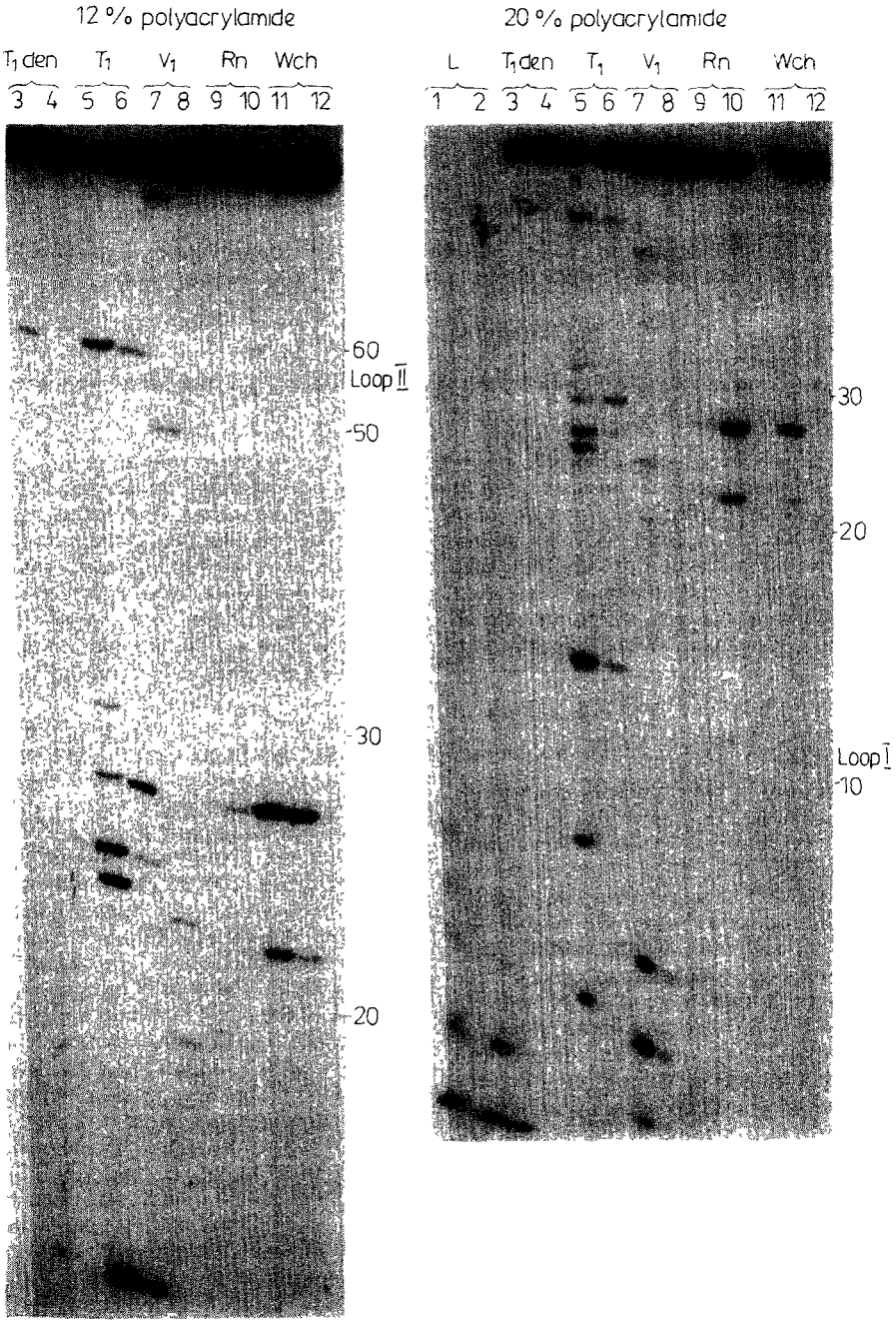

Fig 1 Limited digests of CopA RNA with nucleases Rn, Wch, T1 and V1 1,2—alkaline ladder, 3,4—T1 under denaturing conditions, 5,6—T1 under nondenaturing conditions The numbers and letters indicate the bases connected from the 5'-terminal A in the CopA RNA sequence

[5]. The double-stranded stem II is not cleaved by Rn nuclease, but the enzyme can recognize and cleave the bulged-out U52 near loop II Rn nuclease cleaves phosphodiester bonds in the second loop of the CopA RNA molecule with the preference at positions 56–58. The 3' end of the CopA RNA molecule was not visible when using this version of the method The above cleavage data are summarized in Fig. 2.

Some nucleases used for RNA structure studies sometimes give secondary cuts as a result of what is called 'overcutting'. To see how big the risk of 'overcutting' is in the case of the Rn enzyme, we carried out experiments with a higher concentration of the nuclease and longer incubation times Using a 10 times higher concentration of the enzyme and incubation times up to 3 hr we did not observe any secondary cuts Thus, the risk of 'overcutting' when using Rn nuclease seems to be very small. The RNA secondary structure can be affected by buffer conditions It is therefore important to study the RNA structure under conditions where the molecule is biologically active.

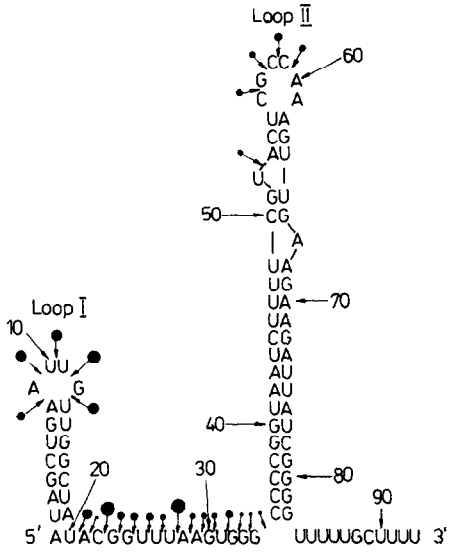

Fig 2. Cleavage positions introduced by Rn nuclease into CopA RNA molecule Preferred cleavage positions are indicated by larger symbols.

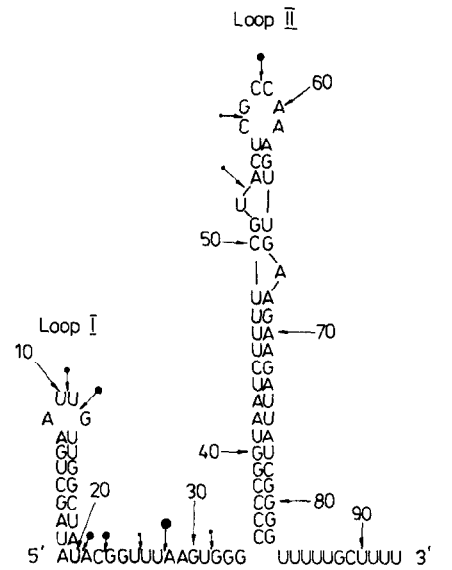

Fig. 3 Cleavage positions introduced by Wch nuclease into CopA RNA molecule. Preferred cleavage positions are indicated by larger symbols.

TMN buffer commonly used for RNA secondary structure studies [7] is one of the buffers in which CopA RNA is active We incubated CopA RNA with Rn nuclease in TMN buffer and analysed the reaction products. The obtained cleavage patterns were identical with those obtained in previous experiments confirming the useful effect of Rn nuclease in RNA structure studies with the use of buffer TMN.

Limited digestion of CopA RNA with Wch nuclease

CopA RNA was subjected to partial digestion with single-strand-specific Wch nuclease and the products of reaction were analysed as above. We established that Wch nuclease is base-specific and shows a strong preference for pyrimidines in non-base-paired regions of RNA (Figs 1 and 3) The enzyme recognizes and cleaves at both uridine residues in the loop I (U10 and U11). In the single-stranded spacer region Wch nuclease cleaves at all pyrimidines with strong preference for C22 and U27. We observe also a weak cutting at U20. Loop II is recognized by the enzyme and cleaved predominantly at C56 and C58 Double-stranded stems I and II are protected from Wch nuclease digestion, but the enzyme cleaves bulged-out U52. Wch enzyme gives the same cleavage pattern acting in the presence of TMN buffer.

DISCUSSION

Two single-strand-specific nucleases from higher plants: Rn nuclease from rye germ nucleoplasm and Wch nuclease from wheat chloroplasts were shown to be good tools for RNA secondary structure investigation. Rn nuclease seems to be particularly useful for such studies. This enzyme is sugar-non-specific and can recognize all phosphodiester bonds in non-base-paired regions of RNA. On the other hand, double-stranded regions of RNA are completely resistant to the nucleolytic attack of this nuclease. Thus, using just one enzyme—Rn nuclease, one can easily map the secondary structure of the RNA molecule. The data concerning the secondary structure of RNA obtained with Rn nuclease summarizes nearly all information obtained earlier by Wagner and others using several different enzymes [5]. Rn enzyme seems to be very precise and can recognize the single bulged-out U52 near loop II, which was shown to be at least partially non-base-paired [5]. In contrast, another single-strand-specific enzyme, nuclease T2, does not recognize this site as single-stranded [5]. Cleavage behind position A8 by the Rn enzyme confirms the earlier observation, that this residue is non-base-paired, at least to some extent. This position is accessible to T2 nuclease attack [5]. Rn nuclease cuts like RNase A, at G12, confirming that this residue belongs to loop I in the CopA RNA molecule. G12 position, however, was not recognized by single-strand-specific nuclease T2. Both, nuclease Rn and ribonuclease A recognize position U20 as single-stranded (possible breathing region). The Rn enzyme cuts very poorly at A21, which is recognized by double-strand-specific V1 nuclease and possibly involved in tertiary interactions Rn nuclease acts on single-stranded RNA very specifically and the risk of secondary cuts by Rn enzyme is very small. This was specially visible in experiments in which the product for Rn enzyme was RepA mRNA (data not shown). Rn nuclease have introduced into the mRNA molecule discrete cuts in definite places independently of enzyme concentration, whereas other structure-specific enzymes cut mRNA almost at random. One of those single-stranded regions was identified as a region complementary to the bigger loop of CopA RNA (data not shown). In all experiments Rn nuclease was incubated with the substrate at 37° and for a rather long time 30 min to 3 hr. Other structure-specific enzymes used in those experiments require very low temperatures and short incubation times to reduce the risk of 'over-cutting' (incubation mixtures kept on ice). Rn nuclease as well as Wch nuclease can act at a pH range close to neutral, in contrast to S1 nuclease commonly used for

secondary structure study of nucleic acids The low optimum pH range (4.0 to 4.3) of S1 nuclease requires that the reaction mixture be sufficiently acidic to cause appreciable depurination of nucleic acid in extended digests.

Rn and Wch nucleases are also active in TMN buffer, which is commonly used for RNA structure investigation. In this buffer the CopA RNA molecule maintains its biological activity. Rn nuclease is very stable and when frozen can retain its activity for several years. Wch nuclease from wheat chloroplasts is also a good tool for secondary structure study This enzyme is base-specific and recognizes all pyrimidines in non-base-paired regions

EXPERIMENTAL

Plant material The rye (*Secale cereale* L) germs were obtained from Swiebodzin Mills (Poland) Wheat (*Triticum vulgare* L var 'Mironowskaya') grains were surface-sterilized with 0 1% w/v $HgCl_2$ aq soln and rinsed with H_2O The grains were imbibed in tap H_2O for 2 hr and then grown on sterilized lignin moistened with H_2O for 7 days at room temp under a normal light regime

Plasmid Plasmid pKN505 carrying the CopA gene of wild type plasmid R1 was isolated as described in ref [5] Chemicals ^{32}P-ATP 3000 Ci/mmol was a New England Nuclear product *Escherichia coli* RNA polymerase, T1 and V1 nucleases were produced by P-L Biochemicals RNAsin was from Promega Biotech RNase-free DNase, phosphoenol pyruvate and pyruvate kinase were Sigma products

Isolation of rye germ nuclei Nuclei from rye germs were isolated as described in ref [1]

Preparation of wheat chloroplasts Chloroplasts were isolated from wheat leaves according to ref [2]

Purification of Rn nuclease Single-strand-specific nuclease from rye germ nucleoplasm was purified as described in ref [1].

Purification of Wch nuclease Single-strand-specific nuclease from wheat leaves chloroplasts was purified according to ref [2]

Preparation of 5'-end-labelled CopA RNA ^{32}P-5'-end-labelled CopA RNA was synthesized *in vitro* using pKN505 plasmid DNA according to ref [5]

Partial digestion of CopA RNA with Rn nuclease The reaction mixture contained 1 5 μl of ^{32}P-end-labelled CopA RNA solution in TMN buffer (20 mM Tris–OAc pH 7 5, 10 mM $Mg(OAc)_2$, 100 mM NaCl), 1 5 μl of Rn enzyme (0 025 units) in 0 01 M NaOAc buffer pH 5.8 and 1 5 μl of TMN buffer or 1 5 μl of 0 1 M NaOAc buffer containing 20 mM $MgCl_2$, pH 6 5 The final reaction vols were 4 5 μl and carrier tRNA was present at 70 μg/ml Reactions were run for 1–3 hr at 37° and stopped by the addition of 4 5 μl stop soln (9 M urea, 20 mM Na_2EDTA, Xylene Cyanol 0 05%, Bromophenol Blue of 0 05%) and heating to 100° for 3 min before placing on ice

Partial digestion of CopA RNA with Wch nuclease The reaction mixture contained 1 5 μl of radioactive CopA RNA soln in TMN buffer, 1 5 μl of Wch enzyme (2×10^{-4} to 2×10^{-3} units) in 0 01 M Tris–HCl buffer, pH 7 4 and 1 5 μl of TMN buffer, pH 7.5 Carrier tRNA was present at 70 μg/ml. Reactions were run for 0 5 to 1 hr at 37° and stopped as described for Rn enzyme

Partial digestion of CopA RNA with T1 and V1 nuclease Enzyme digests were performed in TMN buffer for 30 min on ice at T1 nuclease concn of 5×10^{-4} to 0 1 units/μg RNA at V1 nuclease concn of 1×10^{-3} to 0 5 units/μg RNA Enzymes were diluted in TMN buffer The reactions were stopped as described above

CopA RNA was digested with ribonuclease T1 under denaturing conditions as described in ref [8]

Limited alkaline hydrolysis ^{32}P-5'-end-labelled CopA RNA was incubated in 50 mM NaOH, 1 mM Na_2EDTA at 100° for 15 sec The reaction was stopped by the addition of an equal vol of neutralization buffer (9 5 M urea, 50 mM NaOAc, pH 4 5) and placing the samples on ice

Gel electrophoresis Samples were run on 12 or 20% polyacrylamide gels containing 7 M urea and autoradiographed as described [5]

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