Structural specificity of Rn nuclease I as probed on yeast tRNA\(^{\text{Phe}}\) and tRNA\(^{\text{Asp}}\)

A. Przykorska, C. EI Adlouni\(^1\), G. Keith\(^1\)\(*\), J.W. Szarkowski and G. Dirheimer\(^1\)

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Rakowiecka 36, 02-532 Warszawa, Poland and \(^1\)Institut de Biologie Moléculaire et Cellulaire du Centre National de la Recherche Scientifique et Université Louis Pasteur, 15 Rue Descartes, 67084 Strasbourg, France

Received December 31, 1991; Accepted January 22, 1992

ABSTRACT

A single-strand-specific nuclease from rye germ (Rn nuclease I) was characterized as a tool for secondary and tertiary structure investigation of RNAs. To test the procedure, yeast tRNA\(^{\text{Phe}}\) and tRNA\(^{\text{Asp}}\) for which the tertiary structures are known, as well as the 3'-half of tRNA\(^{\text{Asp}}\) were used as substrates. In tRNA\(^{\text{Phe}}\) the nuclease introduced main primary cuts at positions U33 and A35 of the anticodon loop and G18 and G19 of the D loop. No primary cuts were observed within the double stranded stems. In tRNA\(^{\text{Asp}}\) the nuclease introduced main primary cuts at positions U33, G34, U35, C36 of the anticodon loop and G18 and C20:1 positions in the D loop. No cuts were observed in the T loop. In intact tRNA\(^{\text{Asp}}\) but strong primary cleavages occurred at positions +55, C56, A57 within that loop in the absence of the tertiary interactions between T and D loops (use of 3'-half tRNA\(^{\text{Asp}}\)). These results show that Rn nuclease I is specific for exposed single-stranded regions.

INTRODUCTION

Rn nuclease I from rye germ nuclei (1) has been previously shown to recognize single-stranded regions in native RNA molecules (2, 3). However, the tertiary structures of the RNAs, which were previously used as substrates, were not known. To better characterize the substrate specificity of Rn nuclease I, in order to use it as a tool for structural studies, we have now used tRNA\(^{\text{Phe}}\) and tRNA\(^{\text{Asp}}\) from yeast, two tRNA species for which the crystallographic (4–7) and solution structures have been well characterized (8–16).

RESULT AND DISCUSSION

Yeast tRNA\(^{\text{Phe}}\) as a Rn nuclease I substrate

As was previously shown by Keith and Dirheimer (17), there are two species of tRNA\(^{\text{Phe}}\) in yeast which differ in primary structure by one base pair substitution. In tRNA\(^{\text{Phe}}_{\text{m}}\) (m for minor because it represents only 20% of the total tRNA\(^{\text{Phe}}\)) there is a G-C base pair at positions 67 and 6 whereas the tRNA\(^{\text{Phe}}_{\text{M}}\) (M for major) has a A-U base pair in these positions. These two tRNA\(^{\text{Phe}}\) species were shown to differ significantly in their electrophoretic mobilities under non-denaturing conditions. Because of these structural and electrophoretic mobility differences we studied the nuclease activity on the separate species rather than on the mixture.

Major tRNA\(^{\text{Phe}}_{\text{M}}\)

End 5'-\(^{32}\)P labelled tRNA\(^{\text{Phe}}_{\text{M}}\) was subjected to partial hydrolysis by Rn nuclease I in TMK buffer at 0°C for 10 min. and further analyzed on 20% and 8% PAGE. With the native

MATERIAL AND METHODS

The rye (Secale cereale L.) germ were obtained from the Swiebodzin Mills (Poland). Nuclease I from rye nucleus (Rn) was purified according to ref. (1). Activity of the enzyme preparations was 0.2 units ml\(^{-1}\) (1).

Yeast tRNA\(^{\text{Phe}}_{\text{M}}\) and tRNA\(^{\text{Phe}}_{\text{m}}\) as well as tRNA\(^{\text{Asp}}\) and 3'-half tRNA\(^{\text{Asp}}\) were prepared as previously described (17, 18, 19).

* To whom correspondence should be addressed
5'-[^32]P labelled tRNA[^Phe]m (Fig. 1), two regions were accessible to the Rn nuclease I action: the anticodon loop, and the D loop. The main Rn nuclease cleavages were localised at positions U33 and A35 in the anticodon loop and weaker ones at positions G18 and G19 in the D loop. Much weaker primary cuts at positions A36 and A38 in the anticodon loop and at position G20 in the D loop were observed. The other bands which were seen at positions G15 and U8 corresponded to nonspecific, spontaneous cuts, because they were present in both the control sample and the enzymatic assay.

To be able to discriminate between the so-called primary and secondary cuts (11, 12) enzymatic hydrolyses were done under very limiting conditions (less than one cleavage per tRNA molecule) with the 3'-[^32]P end labelled tRNAs as well. The method used to get the 3'-[^32]P end labelled tRNA was based on removal of the CCA-end by snake venom phosphodiesterase and reconstruction of this sequence in the presence of cold CTP, [α-[^32]P] ATP and tRNA nucleotidyltransferase (22). This method allowed us to work with molecules identical to the native ones rather than extended molecules like those which are obtained by T4 RNA ligase labelling.

The results of limited digestion of 3'-[^32]P labelled tRNA[^Phe]m with Rn nuclease I are shown in Figure 2. The main cuts positions in this case were identical to those found with 5'-[^32]P labelled tRNA[^Phe] (Fig. 1), suggesting that the observed cleavages were primary cuts. The results of these two experiments are summarized on the cloverleaf model of tRNA[^Phe] in Figure 3. The comparison of our results with the known three-dimensional

![Figure 1. Autoradiographs of 20% (A) and 8% (B) PAGE of 5'-[^32]P labelled tRNA[^Phe]m. Lane 1: T1-RNase ladder; lane 2: H2O ladder; lane 3: incubation control; lanes 4 and 5: partial Rn nuclease I digests (0.1 and 0.2 units of enzyme respectively, 0°C, 10 min).](image1)

![Figure 2. Autoradiographs of 20% (A) and 8% (B) PAGE of 3'-[^32]P labelled tRNA[^Phe]m. Lane 1: T1-RNase ladder; lane 2: H2O ladder; lane 3: incubation control; (A) lanes 4–8: partial Rn nuclease I digests (lanes 4 and 5: 0.05 units of enzyme, lanes 6 and 7: 0.1 units of enzyme, lane 8: 0.2 units of enzyme, O°C, 10 min); (B) lanes 4 and 5: 0.1 and 0.2 units of enzyme respectively.](image2)

![Figure 3. Model of the secondary structure of tRNA[^Phe]m. Arrows represent Rn nuclease I cuts. The base pair substitution occurring in tRNA[^Phe]m is shown in brackets.](image3)
structure of tRNA\textsuperscript{Phe} lead us to the conclusion that Rn nuclease recognizes single stranded regions which are exposed to the solvents (11). The same regions are also recognized by other single-strand specific nucleases such as T1-RNase, U2-RNase and S1 nuclease, but none of these enzymes is able to cut all these positions at the same time. For example (i) S1 nuclease cleaves tRNA\textsuperscript{Phe} at positions U33, A35 and A36 in the anticodon loop and at the 3'-end of the tRNA\textsuperscript{Phe}, but not in the D loop (13–15) (ii) T1-RNase introduces main primary cuts at positions G18, G19 and G20 in the D loop (11, 14, 16) thus only after Gs as it is strictly base specific. (iii) U2-RNase is specific for adenines but much less so for guanines; it cleaves tRNA\textsuperscript{Phe} preferentially within the anticodon loop at positions A35, A36 and A38 (11). On the contrary, Rn nuclease I recognizes all the positions at the same time. In the tRNA\textsuperscript{Phe} crystallographic models (4, 5) positions G18 and G19 in the D loop are involved in tertiary interactions with the T loop. Since the same positions are also recognized by Rn nuclease I, the question arises whether the action of Rn nuclease I is hindered by tertiary interactions in RNA molecules or whether these positions are exposed enough to be cleaved by a single-strand-specific enzyme. To answer these questions we have investigated the temperature dependence of the enzymatic reactions on tRNA\textsuperscript{Phe}. The tRNA\textsuperscript{Phe} tertiary structure is stable at moderately elevated temperatures, however tertiary interactions are known to melt at higher temperatures. There were no changes in primary cuts up to about 40°C but some additional cuts appeared especially within the D loop at higher temperatures (for example G19 is better cut at 60°C than at lower temperatures) (Fig. 4). This shows that relaxation of the tRNA's tertiary structure favors the Rn nuclease I action. It allows better accessibility of Rn nuclease I to relaxed regions.

Minor tRNA\textsuperscript{Phe}\textsubscript{m}

Both 5' and 3'-\textsuperscript{[32P]} labelled minor tRNAs\textsuperscript{Phe} were also subjected to the limited Rn nuclease I action and the reaction products were analyzed by PAGE (Fig. 5). The primary cuts which were introduced by the enzyme were exactly at the same positions as those found with tRNA\textsuperscript{Phe}. Therefore, in the native tRNA\textsuperscript{Phe} and tRNA\textsuperscript{Phe}\textsubscript{m}, which are known to differ by a base pair substitution at positions 67 and 6, no differences were found using Rn nuclease I as the structural probe. The graphic modelisation also showed that the introduction of this base pair change does not influence the three dimensional model (P. Dumas, personal communication). Our results are therefore in good agreement with the known crystal structure of tRNA\textsuperscript{Phe} determined on the mixture of the two tRNAs\textsuperscript{Phe} which, despite a difference in primary structure, cocrystallised. Indeed, when the crystallographic studies were done, the existence of the minor tRNA\textsuperscript{Phe} (20% of total tRNAs\textsuperscript{Phe} in yeast) was not known.

tRNA\textsuperscript{Asp} as a Rn nuclease I substrate

Another tRNA of known crystallographic structure, tRNA\textsuperscript{Asp}, was also used to characterize the specificity of Rn nuclease I. Both, 5' and 3'-\textsuperscript{[32P]} labelled tRNA\textsuperscript{Asp} were hydrolysed and analysed as above (Fig. 6). The corresponding results are summarized in Figure 7. The main primary cuts introduced were localized in the anticodon loop at positions U33, G34, U35, C36 and in the D loop at several positions (especially at position C20:1). In addition, the 3'-end of the molecule was hydrolysed.

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**Figure 4.** Temperature dependence of the Rn nuclease I action on tRNA\textsuperscript{Phe}\textsubscript{M}. Lane 1: T1-RNase ladder; lane 2: \textsubscript{H}2O ladder; lane 3: incubation control; lanes 4 and 5: 0.05 units of enzyme, 0°C; lanes 6 and 7: 0.1 units of enzyme 0°C; lane 8: 0.2 units of enzyme, 0°C; lane 9: 0.1 units of enzyme 20°C; lane 10: 0.1 units of enzyme 37°C; lane 11: 0.1 units of enzyme 60°C; lane 12: 0.1 units of enzyme 75°C; lane 13: 0.1 units of enzyme 85°C.

**Figure 5.** Autoradiographs of 20% (A) and 8% (B) PAGE of 3'-\textsuperscript{[32P]} labelled tRNA\textsuperscript{Phe}. Lane 1: T1-RNase ladder; lane 2: \textsubscript{H}2O ladder; lane 3: incubation control; lanes 4–6: partial Rn nuclease I digests (A—lanes 4 and 5: 0.2 and 0.1 units of enzyme respectively, B—lanes 4 and 5: 0.2 of enzyme, lane 6: 0.1 units of enzyme, 0°C, 10 min).
However in the electrophoretical conditions which were used, that region is only poorly visible. The regions of native tRNA\textsuperscript{Amp} molecules which were recognized by Rn nuclease I are known from crystallographic studies to be exposed and were also cleaved by other single-strand-specific nucleases, but not all of them by a single enzyme at the same time. For example S1 nuclease cuts tRNA\textsuperscript{Amp} at positions U33, G34, U35 and C36 within the anticodon loop and at positions C74, C75 and A76 close to the 3' end, but does not cut within the D loop (11). T1-RNase cuts in the anticodon loop only at position G34 as well as at positions G18 and G19 in the D loop and at G73 at the 3'-end of the molecule (11).

The temperature dependance of the enzymatic hydrolysis of tRNA\textsuperscript{Amp} by Rn nuclease I showed, that at moderately high temperatures, when partial denaturation of the tRNA may have already occurred but the enzyme still active (it is active < 80°C), some very weak additional cuts took place (data not shown). However, tRNA\textsuperscript{Amp} molecules are very stable up to 70°C. It is

![Figure 6](image1.png)

**Figure 6.** Autoradiographs of 20% (A) and 8% (B) PAGE of 3'-[\textsuperscript{32}P] labelled tRNA\textsuperscript{Amp}. Lane 1: T1 RNase ladder; lane 2: H\textsubscript{2}O ladder; lane 3: incubation control; lanes 4 and 5: partial Rn nuclease I digests (0.2, and 0.1 units of the enzyme respectively, 0°C, 10 min). (C) Anticodon loop cleaved by Rn nuclease I. Lane 1: incubation control, lanes 2, 3 and 4: 0.1, 0.2 and 0.3 units of enzyme respectively.

![Figure 7](image2.png)

**Figure 7.** Model of the secondary structure of yeast tRNA\textsuperscript{Amp}. Arrows represent Rn nuclease I cuts.

![Figure 8](image3.png)

**Figure 8.** Autoradiograph of 20% PAGE of 5'-[\textsuperscript{32}P] labelled 3'-half of tRNA\textsuperscript{Amp}. Lane 1: T1-RNase ladder; lane 2: H\textsubscript{2}O ladder; lane 3: incubation control; lanes 4-6: partial Rn nuclease I digests (0.1, 0.05 and 0.2 units of the nuclease I respectively, 0°C, 10 min).

![Figure 9](image4.png)

**Figure 9.** Model of the secondary structure of the 3'-half tRNA\textsuperscript{Amp}. Arrows represent Rn nuclease I cuts.
thus very difficult to selectively remove tertiary interactions by increasing the temperature (23).

3′-Half of tRNA<sup>Asp</sup> as a Rn nuclease I substrate

In order to remove the tertiary interactions between the D and T loops we have prepared the 3′-half of the tRNA<sup>Asp</sup> molecule. This fragment is known to maintain its secondary structure as well as intrinsic interactions even without the structural context of the entire tRNA molecule (24). Both 5′- or 3′-[<sup>32</sup>P]labelled 3′ half tRNA<sup>Asp</sup> preparations were subjected to limited Rn nuclease I hydrolysis. Strong primary cuts were introduced at positions 55, 56 and A57 whereas positions A58, U59, and U60 remained resistant to Rn nuclease I (Fig. 8). It is interesting to notice the dramatic difference in accessibility between these positions. This difference is in good agreement with the known conformation of the 3′-half of tRNA<sup>Asp</sup> (13). Indeed, it has been shown that phosphate 60 in the 3′-half of tRNA<sup>Asp</sup> is engaged in hydrogen bonding with C61 and the ribose of A58 as it is in the entire tRNA<sup>Asp</sup> molecule. A58 is also engaged in reverse Hoogsteen base pairing with T54 as it is in the intact molecule. The other positions which were accessible for Rn nuclease I in the 3′-half of tRNA<sup>Asp</sup> are nucleotides U35 to G59 and G71 to C75 (not shown). Some weaker cuts were observed in the region between U40–U47 (Fig. 8). No cuts were observed in the T stem, which is known to be very stable (19, 23). These results are in good agreement with the proposed secondary structure model for the 3′-half of yeast tRNA<sup>Asp</sup> (24) in which the T-arm is extended by interactions between the 3′ and 5′-end of the fragment (Fig. 9). This model which contains bulged nucleotides is extended by interactions between the 3′-half of yeast tRNA<sup>Asp</sup> and includes a dynamic behavior of its pseudo-helical conformation which could thus be confirmed by the accessibility of some nucleotides to the Rn nuclease I reaction.

In summary, using well known and reliable substrates we have shown that Rn nuclease I from rye germ nucleus can be a very useful tool for the secondary structure studies of native RNA molecules in solution. The enzyme is very stable and easy to use. It works well under the conditions which are commonly used for RNA structure studies (TMK buffer, pH 7.5). In addition, Rn nuclease I seems to be much more accurate in RNA secondary structure studies than the other enzymes usually used in that field because it gives the same information about RNAs structure in one step, for which several different enzymes, commonly used in structural studies, were previously needed.

ACKNOWLEDGMENTS

This work was supported by a short term FEBS fellowship to A. P. and by the 'Jumelage programme' between the Centre National de la Recherche Scientifique and the Polish Academy of Sciences. We are indebted to P. Romby, C. Ehresmann and B. Ehresmann for stimulating discussions, to P. Dumas for graphic modelisation and to B. Winsor for improving the English.

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